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Comparative Characterization of Phosphoenolpyruvate Carboxylase in C_3, C_4, and C_3–C_4 Intermediate *Panicum* Species[1]

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ABSTRACT

Various properties of phosphoenolpyruvate carboxylases were compared in leaf preparations from C_3–C_4 intermediate, C_3, and C_4 *Panicum* species. Values of V_{max} in micromoles per milligram chlorophyll per hour at pH 8.3 were 57 to 75 for the enzyme from *Panicum milioides*, *Panicum schenckii*, and *Panicum decipiens* (all C_3–C_4). The values for *Panicum laxum* (C_3) and *Panicum prionitis* (C_4) were 20 to 40 and 952 to 1374, respectively. The V_{max} values did not change at pH 7.3 except for the C_4 value, which increased about 24%. At pH 8.3, the phosphoenolpyruvate carboxylases from C_3 and C_3–C_4 species had slightly higher K_m HCO_3^- and lower K' phosphoenolpyruvate values than did the C_4 enzyme. With each species at pH 7.3, all K' phosphoenolpyruvate values were 2- to 4-fold greater.

The enzyme from all species was inhibited 85 to 90% by 1 millimolar malate at rate-limiting phosphoenolpyruvate and Mg^{2+} levels. With low levels of malate, 0.2 millimolar, the rate curve with respect to phosphoenolpyruvate was distinctly sigmoidal, and the inhibition was not eliminated at 5 millimolar phosphoenolpyruvate.

Malate at 10 millimolar protected all phosphoenolpyruvate carboxylases from inactivation at 55 C at pH 5.5, but not at pH 8.3. Aspartate did not protect well. When incubated at 37 C at pH 8.3 without phosphoenolpyruvate, but with HCO_3^-, the enzyme from several C_4 grasses lost 92 to 98% of the initial activity after 4 minutes, whereas the enzymes from C_3 and C_3–C_4 *Panicum* species retained 60 to 70% of their activities. In contrast, 5 millimolar phosphoenolpyruvate stabilized the enzyme at 37 C in all plant extracts.

The phosphoenolpyruvate carboxylase from C_3–C_4 intermediate *Panicum* species has properties most similar to the enzyme from C_3 *Panicum* species. The higher leaf activity of the enzyme from the intermediate plants than from C_3 species is not due to any unusual property assayed other than a higher V_{max}.

Different forms of PEP[2] carboxylase are present in green tissues of C_3 and C_4 photosynthesis plants. *In vitro*, these forms differ principally in kinetic properties, inactivation by heat, and in susceptibility to inhibitors and activators. These distinguishing characteristics can be related to the roles of PEP carboxylase in specific tissues.

In terms of HCO_3^-, the kinetic data for all leaf PEP carboxylases can be analyzed using the Michaelis-Menten model and many reports indicate low K_m values (10, 14, 20, 22). However, the model cannot be used for kinetic data with respect to PEP (20). Because of the apparent allosteric nature of PEP carboxylase, the Hill equation (19) has been used to derive certain kinetic constants with respect to PEP, and these properties were used to define different forms of the enzyme in leaves with different pathways of photosynthetic carbon metabolism. With a C_3 *Atriplex* species, the PEP carboxylase K' (PEP) value was 80 μM, but a C_4 *Atriplex* species K' was 0.49 mM (20).

Heat inactivates the enzyme but, reputably, the C_4 enzyme is protected from heat inactivation at 55 C by 10 mM malate or aspartate, whereas the C_3 enzyme is not (17). In effector studies, at rate-limiting levels of PEP and Mg^{2+}, the C_3 enzyme is slightly inhibited (8% or less) by 1 mM malate or aspartate, but the C_4 enzyme is inhibited as much as 66% (8). Glucose-6-P increases the activity of the partially purified enzyme from C_4 and CAM species (4, 21–23) at saturating substrate and Mg^{2+} concentrations, whereas glycine activates only the enzyme from monocot C_4 species (16, 22).

Recently, *Panicum milioides*, a grass with photorespiratory activity intermediate between C_3 and C_4 plants (2), was reported to possess a C_4 photosynthesis system with PEP carboxylase responsible for 24% of the initial CO_2 assimilation (18). However, earlier photosynthesis work in our laboratory using room air failed to detect ^{14}C in organic acids, therefore indicating a low *in vivo* PEP carboxylase activity (5, 9), as found in C_3 species. But PEP carboxylase is present in C_3–C_4 intermediate species at levels above those in C_3 plants (9). The genus *Panicum* contains C_3, C_4, and C_3–C_4 intermediate species. The study presented here was initiated to determine whether PEP carboxylase in the intermediate *Panicum* species has properties similar to those of either the C_3 or the C_4 enzyme or if the enzyme in intermediate *Panicum* species has unique properties which indicate a role in the reduction of photorespiration in C_3–C_4 intermediate plants.

MATERIALS AND METHODS

Plant Material. Mature leaves were obtained from greenhouse-grown plants of *P. milioides* Nees ex Trin. (C_3–C_4), *Panicum schenckii* Hack. (C_3–C_4), *Panicum decipiens* Nees ex Trin. (C_3–C_4), *Panicum laxum* Sw. (C_3), *Panicum prionitis* Griseb. (C_4), *Digitaria sanguinalis* (L.) Scop. (C_4), and *Zea mays* (L.) (C_4). These *Panicum* species are members of the subgroup *Laxa* (7) and possess photosynthetic characteristics of the types indicated in parentheses above (2, 3, 11, 12).

Enzyme Assays. Except for the effector studies, the PEP carboxylase assays were performed using whole-leaf extracts prepared by grinding 0.1 g fresh weight of leaf material as previously described (23). Aliquots were taken for Chl determination (1) before centrifugation. PEP carboxylase activity was determined (unless otherwise specified) at 30 C by the ^{14}C incorporation method of Van *et al.* (23). The assay solutions contained, in 1 ml: 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 5 mM DTT, 10 mM $NaH^{14}CO_3$ (0.5 μCi/μmol), 5 mM PEP, and plant extract.

In the kinetic studies, the concentration of HCO_3^- or PEP was varied. For the HCO_3^- analyses, a "CO_2-free" Tris-HCl (pH 8.3) buffer solution was used and all reactions were performed in

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[2] Abbreviation: PEP, phosphoenolpyruvate.

sealed reaction vessels purged of CO_2 before addition of the buffer. For studies at pH 7.3, the assays also were conducted in sealed vessels, but contained 100 mM Tris-HCl buffer. The duration of the reactions was 1 min or less, during which time the rate of PEP carboxylation was linear.

Effector Studies. The effects of glucose-6-P and glycine (0.1 to 5.0 mM) on PEP carboxylase activity were determined under the enzyme assay conditions and grinding procedures described above (24). The effect of L-malate (0.02 to 2.0 mM) at rate-limiting substrate concentrations was determined on extracts prepared in a similar manner except that the grinding solution contained 50 mM Tris-HCl (pH 7.5) and 2 mM $MgCl_2$. The extracts used in all studies were passed through a Sephadex G-25 column equilibrated with the grinding solution.

Assay solutions for the malate study contained, in 1 ml: 50 mM Tris-HCl or 50 mM Tricine-NaOH (pH 7.5), 2 mM $MgCl_2$, 5 mM DTT, 0.9 mM PEP, 10 mM $NaH^{14}CO_3$ (0.5 μCi/μmol), 0.9 mM PEP and various concentrations of L-malate added as a solution buffered at pH 7.5 or 0.2 mM malate (pH 7.5) and various concentrations of PEP added as buffered solutions, and up to 0.2 ml of the plant extract. Reactions were terminated within 30 s by the addition of 0.1 ml of a 6 N HCl solution saturated with 2,4-dinitrophenylhydrazine, and the level of ^{14}C fixation was determined (23).

Heat Inactivation. The ability of L-malate and L-aspartate to protect PEP carboxylase from heat inactivation was examined. Leaf extracts from *P. laxum*, *P. milioides*, and *P. prionitis* were incubated at 0 or 55 C either at pH 5.5 or 8.3 for 10 min with either 10 mM malate or 10 mM aspartate, or with no C_4 acid present. Then PEP carboxylase was assayed at 30 C and pH 8.3 on the treated extracts as described (23).

The forms of PEP carboxylase in different species also were found to differ in their stability when incubated without PEP at 37 C in the complete assay medium (23). Leaf extracts were incubated for several min at 37 C minus PEP, and then PEP carboxylase activity was measured at 37 C by adding 5 mM PEP.

RESULTS AND DISCUSSION

Kinetics with Respect to HCO_3^-. Double-reciprocal plots of the rate of carboxylation *versus* the added HCO_3^- concentration indicated that the data followed the Michaelis-Menten model (data not shown). The V_{max} values for PEP carboxylase from *P. milioides* were higher than those for *P. laxum* (Table I). We previously reported higher PEP carboxylase activity in C_3–C_4 intermediate *Panicum* species than was found in most C_3 plants (5, 9, 12). These reports led to the hypothesis that PEP carboxylase is important in reducing photorespiration and O_2 inhibition of photosynthesis in C_3–C_4 intermediate species (5, 9). Yet the V_{max} of PEP carboxylase from *P. milioides* is not nearly as great as the V_{max} of the enzyme from the C_4 plant *P. prionitis*, in which it is the principal carboxylating enzyme using ambient CO_2 (12) (Table I).

Previous studies with many other species indicate that K_m values for HCO_3^- for PEP carboxylase generally are low (~0.02 to 0.10 mM (14, 20, 22). The K_m values for the three *Panicum* species are higher (0.4 to 0.8 mM) (Table I). K_m values greater than 0.10 mM HCO_3^- also have been reported in other species (4, 13, 15); thus, K_m values for HCO_3^- are not consistently low in green tissues. When one considers the variation in the data, the K_m values determined for the enzyme from *P. laxum* and *P. milioides* are only slightly higher than those for the *P. prionitis* enzyme (Table I).

Table I. *Range of V_{max} and K_m Values for HCO_3^- of PEP Carboxylase from Three Panicum Species*

Values were calculated from double-reciprocal plots of velocity *versus* added HCO_3^- concentration.

Species	V_{max}	K_m (HCO_3^-)
	μmol CO_2/mg Chl·h	*mM*
P. milioides	57–69	0.4–0.8
P. laxum	21–39	0.4–0.8
P. prionitis	1374	0.3

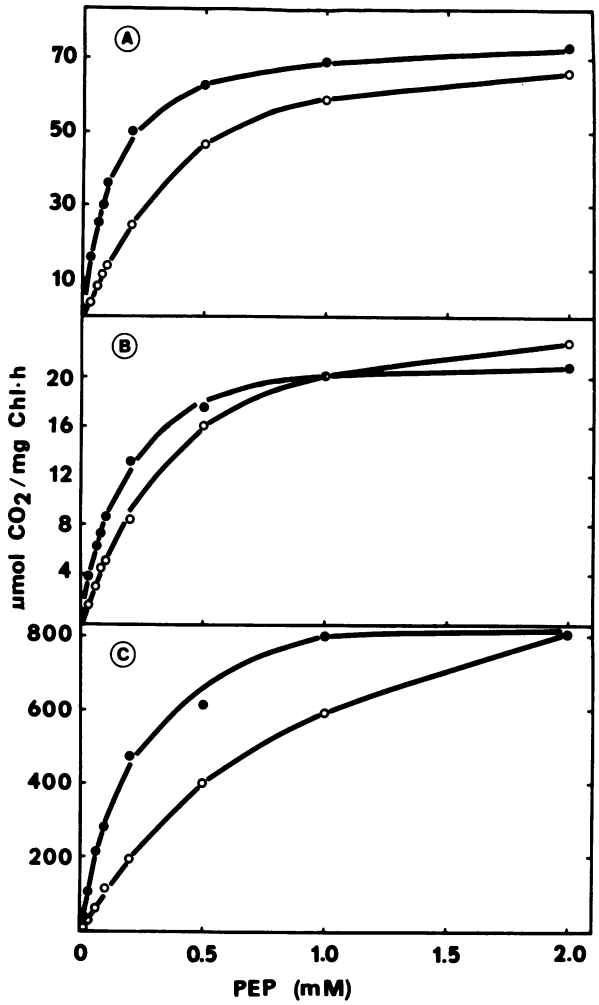

FIG. 1. PEP carboxylase velocity *versus* PEP concentration at pH 8.3 (●) and pH 7.3 (○) for: A, *P. milioides*; B, *P. laxum*; and C, *P. prionitis*.

Kinetics with Respect to PEP. Ting and Osmond (20) reported that rate curves with respect to PEP determined at pH 7.8 for PEP carboxylase from C_4 plants exhibited sigmoidal characteristics indicative of an allosteric enzyme, whereas those curves for the enzyme from C_3 plants appeared more hyperbolic. The rate curve determined at pH 8.3 for PEP carboxylase in *P. milioides* does not show any sigmoidal characteristics (Fig. 1A). Similar curves also were obtained for the enzyme from the intermediates *P. schenckii* and *P. decipiens* (data not shown) and for the PEP carboxylase from *P. laxum* (Fig. 1B). The curve for the C_4 enzyme from *P. prionitis* (Fig. 1C) also exhibited no sigmoidal characteristics as found by Murkerji (13) for *Z. mays* PEP carboxylase.

Uedan and Sugiyama (22) reported that, in the presence of organic activators or at pH 8, the partially purified PEP carboxylase from *Z. mays* exhibited a hyperbolic rate curve. At pH 7, or in the absence of activators, the curve was somewhat sigmoidal and indicated a lower V_{max} and higher K'_{PEP} for the enzyme. Thus, rate curves for the PEP carboxylase in extracts for *P. milioides*, *P. laxum*, and *P. prionitis* were developed from data obtained at pH 7.3 (Fig. 1, A, B, and C, respectively) to compare with the data at pH 8.3.

The rate curves determined at pH 7.3 for the PEP carboxylase from *P. milioides* and *P. laxum* indicated that enzymic activities saturated at about 2 mM PEP and were similar to those determined at pH 8.3 (Fig. 1, A and B). However, the enzyme from *P. prionitis* at pH 8.3 was nearly saturated at 1 and 2 mM PEP, whereas, at pH 7.3, saturation apparently had not been reached at 2 mM even though the activity was similar to that at pH 8.3 (Fig. 1C).

In spite of the apparent hyperbolic nature of the rate curves in Figure 1, the data did not adhere to Michaelis-Menten kinetics as Ting and Osmond (20) noted. Thus, V_{max} values were derived from double reciprocal plots of the velocity *versus* PEP concentration (0.1 to 2 mM PEP) and the Hill equation (19) was used to determine K' values for PEP.

Values of V_{max} determined at pH 8.3 for the enzyme from three intermediate *Panicum* species were all greater than that for the *P. laxum* PEP carboxylase (Table II). However, the values were not nearly as great as that for the PEP carboxylase from *P. prionitis*. There was little effect of pH on the calculated V_{max} values for the enzyme from *P. milioides* and *P. laxum*, but the V_{max} for the enzyme from *P. prionitis* at pH 7.3 was 24% greater than that at pH 8.3 (Table II). Hatch and Oliver (6) reported that extraction and incubation of *Z. mays* PEP carboxylase at pH 6.9 also increased its subsequent activity.

As found for other C_3 PEP carboxylase forms (20), the K' (PEP) determined at pH 8.3 for the enzyme from *P. laxum* was low (0.06 mM PEP) (Table II). Similarly, the values for PEP carboxylases from the intermediate *Panicum* species were low. As is typical of C_4 PEP carboxylases (20), the K' (PEP) for the enzyme from *P. prionitis* was about 3-fold higher than the C_3 PEP carboxylase value (Table II). The Hill coefficients were 0.92 for the PEP carboxylase from *P. prionitis*, 0.90 to 1.10 for the enzyme from the intermediate species, and 1.33 for the enzyme from *P. laxum*. These values were consistent with the apparent hyperbolic nature of the rate curves determined at pH 8.3 (Fig. 1).

The curves in Figure 1 indicate that, as pH decreased from 8.3 to 7.3, there was an increase in the K' (PEP) for all PEP carboxylases. A 2-fold increase was shown by Uedan and Sugiyama (22) with the *Zea* enzyme. Here, a 2.7-, 3.4-, and 4.2-fold increase in K' (PEP) at pH 7.3 was found for PEP carboxylase from *P. laxum*, *P. milioides*, and *P. prionitis*, respectively. Therefore, the PEP carboxylase in different plants responded differently to these changes in pH, with the C_4 enzyme being the most sensitive to pH changes (Table II).

Thus, the data from these HCO_3^- and PEP kinetic studies indicate that the enzyme from the intermediate *Panicum* species has properties most similar to the C_3 *Panicum* PEP carboxylase.

Effector Studies. Certain investigators (4, 21, 22) reported that 5 mM glucose-6-P increased the activity of partially purified PEP carboxylase from C_4 and CAM species at saturating substrate and Mg^{2+} levels. Also, glycine reportedly activated the enzyme from C_4 grasses (16, 22). Our preliminary analyses (data not shown) indicated no activation of PEP carboxylase from desalted extracts of *P. prionitis*, *P. milioides*, *P. laxum*, and *Z. mays* by glycine or glucose-6-P in the range of 0.1 to 5.0 mM in the presence of 5 mM PEP and 10 mM $MgCl_2$. Therefore, other effector experiments were performed to characterize the PEP carboxylases in *Panicum* species.

Huber and Edwards (8) reported that the C_4 PEP carboxylase was inhibited 14 to 66% by 1 mM malate, and to a lesser degree by aspartate, at pH 7.5 and rate-limiting levels of PEP and Mg^{2+}. The C_3 enzyme exhibited little inhibition. Here, the PEP carboxylases from *P. prionitis*, *P. milioides*, and *P. laxum* were inhibited similarly at 1 mM malate and throughout much of the malate concentration range examined (Fig. 2). Aspartate inhibition was found to be 20 to 30% less (data not shown). Only at 0.1 and 0.2 mM malate did the *P. laxum* PEP carboxylase show somewhat less inhibition (Fig. 2). The 85 to 90% inhibition at 1 mM malate (Fig. 2) was much greater than that reported by Huber and Edwards and was not decreased, even when a 50 mM Tricine-NaOH buffer solution was used (Fig. 2) as in their study (8).

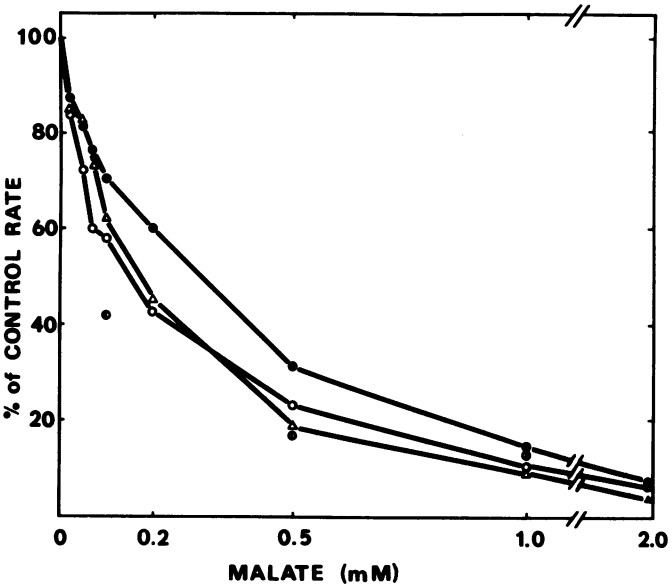

FIG. 2. PEP carboxylase activity *versus* L-malate concentration. (●), *P. laxum*; (○), *P. milioides*; (△), *P. prionitis*. Assay solutions contained 2 mM $MgCl_2$ and 0.9 mM PEP buffered with Tris-HCl (pH 7.5). Points where assay solutions for the *P. milioides* PEP carboxylase were buffered using Tricine-NaOH at pH 7.5 are designated as ⊙.

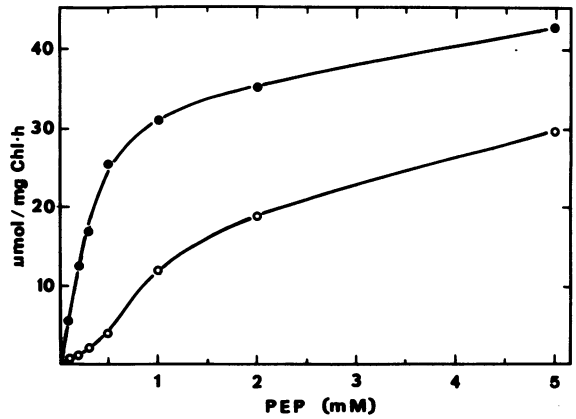

FIG. 3. *P. milioides* PEP carboxylase velocity *versus* PEP concentration in the presence of 0.2 mM malate (○) and without malate (●). The assay solutions contained 10 mM HCO_3^-, 2 mM $MgCl_2$, and 100 mM Tris-HCl (pH 7.5).

Table II. *V_{max} and K' Values for PEP of PEP Carboxylase from Five Panicum Species*

The V_{max} values were calculated from double-reciprocal plots of velocity *versus* PEP concentration (0.1 to 2 mM). The K' (PEP) values were calculated from Hill plots (20).

Species	V_{max}		K' (PEP)	
	pH 8.3	pH 7.3	pH 8.3	pH 7.3
	μmol CO_2/mg Chl·h		mM	
P. milioides	75	73	0.07	0.24
P. decipiens	61		0.09	
P. schenckii	66		0.07	
P. laxum	21	23	0.06	0.16
P. prionitis	952	1176	0.22	0.93

Although the different forms of PEP carboxylase exhibited similar inhibition by malate, their specific activities at a given level of inhibition were markedly different. At 40% inhibition, the PEP carboxylase activities from *P. prionitis*, *P. milioides*, and *P. laxum* were 200, 10, and 6 μmol CO_2/mg Chl·h, respectively. Whereas the contribution to CO_2 assimilation by PEP carboxylase in *P. prionitis* at 40% inhibition would still be considerable, that by the enzyme from *P. milioides* or *P. laxum* would be very low. Thus, to reduce PEP carboxylase activity substantially, much higher levels of malate would be required in the C_4 plant than in the C_3 or the intermediate species.

Huber and Edwards (8) reported that the inhibition due to 1 mM malate could be overcome by increasing the PEP concentration to 3 mM. Thus, the level of PEP in the vicinity of the enzyme would determine the extent of inhibition should the level of malate rise. Yet, the malate inhibition detected in our analysis (Fig. 3) was much greater than that found by Huber and Edwards (8), so a greater concentration of PEP should be required to eliminate the inhibition. Figure 3 shows that, even at 5 mM PEP, the specific activity of the PEP carboxylase from *P. milioides* in the presence of 0.2 mM malate was 30% less than that in the absence of malate. With 0.2 mM malate, the rate curve with respect to PEP was distinctly sigmoidal and indicated an increase in the K' (PEP) as Huber and Edwards (8) reported, but the inhibition at each PEP concentration was greater than they found at 1 mM malate for PEP carboxylase from a C_4 species.

The existence of a PEP concentration of 5 mM or greater in the cytoplasm of *P. milioides* cells is unlikely. The pool size of PEP in leaves of *P. milioides*, although 4-fold greater than in leaves of *P. laxum*, was 3-fold less than in *P. prionitis* (unpublished data). At the *in vivo* PEP carboxylase activity indicated by the data of Rathnam and Chollet (18) and at a cytoplasmic pH of 7 in the mesophyll cells of *P. milioides*, the Mg^{2+} concentration must not be at rate-limiting levels (8) and malate must be removed to avoid substantial inhibition. During steady-state C_4 photosynthesis, C_4 acid movement out of the mesophyll cells is rapid due to a rapid turnover of C_4 acids in bundle-sheath cells. Rathnam and Chollet (18) have reported an even more rapid turnover in *P. milioides* leaves. Considering the *in vitro* sensitivity of the *P. milioides* PEP carboxylase activity to malate (Fig. 3), such a turnover of malate would be essential if 24% of the CO_2 is assimilated by PEP carboxylase in *P. milioides*.

Table III. *Effects of Incubation Temperature in Presence or Absence of Aspartate or Malate at pH 8.3 and 5.5 on PEP Carboxylase from three Panicum Species*

Incubation was for 10 min at 0 or 55 C with aspartate, malate, or no C_4 acid present (control). PEP carboxylase activity then was determined at pH 8.3 and 30 C. Control activities from extracts incubated at 0 C were 664 to 1698, 48 to 56, and 19 to 31 μmol/mg Chl·h for the enzyme from *P. prionitis*, *P. milioides*, and *P. laxum*, respectively.

pH	Species	Incubation Temperature	Control	Aspartate (10 mM)	Malate (10 mM)
		C	% of control (0 C)		
8.3	*P. prionitis*	0	100	113	101
		55	0	0	0
	P. milioides	0	100	112	91
		55	0	0	0
	P. laxum	0	100	116	91
		55	0	0	0
5.5	*P. prionitis*	0	100	86	116
		55	0	8	107
	P. milioides	0	100	64	100
		55	0	13	92
	P. laxum	0	100	110	103
		55	0	32	77

Table IV. *Effects of Various Treatments on Stability at 37 C of PEP Carboxylase from C_4, C_3-C_4 Intermediate, and C_3 Grasses*

The treatment solutions, unless otherwise specified, did not contain PEP and were: Control, assay medium (pH 8.3) containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, and 10 mM $NaH^{14}CO_3$; A, assay medium (pH 7.3); B, assay medium + BSA (6 mg/ml); C, assay medium, but the enzyme extract was first desalted using a Sephadex, G-25 column; D, assay medium with 5 mM PEP.

Species	Treatment Conditions	PEP Carboxylase Activity after Following Incubation Periods			Activity after 4 min
		0 min	1 min	4 min	
		μmol CO_2/mg Chl·h			% of initial
D. sanguinalis	Control	2,838	648	88	3
Z. mays	Control	2,787	1,526	235	8
P. prionitis	Control	1,508	289	29	2
	A	3,778	1,363	338	9
	B	1,287	401	74	6
	C	1,700	286	48	3
	D + PEP − HCO_3^-	1,474	1,212	1,094	74
P. milioides	Control	53	41	33	62
	+ PEP − HCO_3^-	54	50	49	91
P. laxum	Control	32	30	23	71
	+ PEP − HCO_3^-	12	12	11	92

Protection From Heat Inactivation. Rathnam (17) proposed that heat could be used to distinguish different forms of PEP carboxylase and this approach was used to characterize the different forms within *Panicum*. Leaf extracts from *P. laxum*, *P. milioides*, and *P. prionitis* were incubated at pH 5.5 or 8.3 with 10 mM malate or aspartate at 0 or 55 C for 10 min prior to assay at 30 C and pH 8.3. Controls contained no added C_4 acids. Incubation at 55 C resulted in low subsequent PEP carboxylase activities in all controls relative to those for controls incubated at 0 C (Table III). The addition of 10 mM aspartate offered little protection from heat inactivation at either pH 8.3 or 5.5 for the enzyme from *P. prionitis* and *P. milioides*, but 32% of the control activity remained for PEP carboxylase from *P. laxum*. Incubation at 0 C and pH 5.5 with aspartate also resulted in a 36% loss of activity from the enzyme from *P. milioides*.

At pH 8.3, the addition of 10 mM malate offered little protection as well. But, after incubation at 55 C and pH 5.5 with 10 mM malate, the PEP carboxylase activity in the *P. prionitis* extract was 92% of the activity in the 0 C treated extract. The addition of 10 mM malate at pH 5.5 also protected the PEP carboxylase in *P. milioides* and *P. laxum* from heat inactivation with 92 and 77% of the activity, respectively, remaining after 10 min at 55 C (Table III). Rathnam (17) reported that neither C_4 acid protected PEP carboxylases from C_3 species. Apparently, the binding of malate to PEP carboxylase is pH-dependent. This idea is supported by the report that malate inhibition of PEP carboxylase is pH-dependent (8). Unfortunately, Rathnam (17) did not report the pH of his incubated extracts. Therefore, this analysis, as described here, was not adequate to differentiate various forms of PEP carboxylase.

Stability at 37 C. The forms of PEP carboxylase differed in their response to incubation at 37 C in the assay medium, but without PEP. When extracts from three C_4 grasses were incubated for various times, the subsequent PEP carboxylase activity decreased rapidly with increasing time of incubation (Table IV). The carboxylation rate in the presence of PEP and HCO_3^- was constant for at least 2 min (data not shown), but activity was lost during incubation without PEP so that only 2% of the initial activity of the *P. prionitis* PEP carboxylase existed after 4 min. Similarly, two

other C_4 PEP carboxylases lost nearly all of their activity after 4 min. In contrast, the enzyme from *P. laxum* and *P. milioides* retained 71 and 62%, respectively, of the initial activity after 4 min. Thus, the PEP carboxylase from the intermediate plant responded as did the enzyme from the C_3 plant.

Several conditions were studied to determine which stabilized the enzyme from *P. prionitis* at 37 C (Table IV). The addition of BSA (6 mg/ml) to the incubation solution and the lowering of the pH to 7.3, conditions used by Hatch and Oliver (6) to stabilize or activate the *Z. mays* enzyme at 25 or 0 C for 1 to 3 h, had no effect on the loss of activity. Extraction and assay at pH 7.3 substantially increased the initial activity, however. Also, passing the extract through a Sephadex G-25 column before incubation had no effect (Table IV). However, when the enzyme extract was incubated with PEP (without HCO_3^- or CO_2 present) 74% of the initial activity of the enzyme remained after 4 min treatment. The PEP carboxylases from *P. milioides* and *P. laxum* also were more stable when incubated in PEP, retaining 91 and 92% of the initial activity, respectively (Table VI). Thus the *in vivo* level of PEP, and not HCO_3^-, would be critical to PEP carboxylase stability at temperatures often experienced by plants.

CONCLUSIONS

PEP carboxylases from *Panicum* species of different photosynthetic categories exhibit different characteristics. The HCO_3^- and PEP kinetic studies (Fig. 1; Tables I and II) followed patterns reported previously (4, 13, 16, 21) for C_3 and C_4 PEP carboxylases, with the PEP kinetics distinguishing the various photosynthetic forms of PEP carboxylase most clearly. However, except for the V_{max} values being 2- to 3-fold higher than in C_3 *Panicum* species (Tables I and II), no unusual properties of the enzyme from C_3–C_4 intermediate *Panicum* species were found, which were distinct from properties of either the C_3 or C_4 forms.

Apparently, malate protects all forms of the enzyme from inactivation at high temperatures, but only at low (5.5) pH values (Table III). Thus, the discovery that PEP (Table IV), and not HCO_3^-, is essential for stabilizing PEP carboxylase at 37 C and, at pH 8.3, seems important physiologically. When both substrates are present, the enzyme is stable. But, during periods when leaf temperatures are high and HCO_3^- levels may be low (*e.g.* summer droughts), PEP must be present to protect PEP carboxylase from heat inactivation.

Although some analyses (Table III; Fig. 2) failed to segregate the forms of PEP carboxylase, in all situations where there were differences between C_3 and C_4 PEP carboxylases, the enzyme from the intermediate species had characteristics which were most similar to those of the C_3 enzyme (Tables I, II, and IV; Fig. 1). The enzyme from *P. milioides* and other intermediate *Panicum* species is, in these respects, a C_3 PEP carboxylase. The higher leaf activity reported for the intermediate *Panicum* species than that found for most C_3 PEP carboxylases (5, 9) apparently is due to the fact that there is more of the C_3-type enzyme in the intermediate plants and not due to any unusual kinetic properties of that enzyme other than its higher V_{max} (Tables I and II).

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