PHOSPHOENOLPYRUVATE CARBOXYKINASE IN LEAVES OF CERTAIN PLANTS WHICH FIX CO_2 BY THE C_4-DICARBOXYLIC ACID CYCLE OF PHOTOSYNTHESIS*

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SUMMARY: Phosphoenolpyruvate carboxykinase has been found in leaves of *Panicum maximum*, *Panicum texanum*, and *Sporobolus poiretii* at levels sufficient to be involved in photosynthesis. The enzyme preferentially utilizes adenosine nucleotide derivatives. In *Panicum maximum* the enzyme is concentrated in the bundle sheath cells about four times higher than in the mesophyll cells. From 40 to 50 percent of the enzyme activity in bundle sheath cell extracts of *Panicum maximum* can be separated into a particulate fraction. The enzyme may function as a decarboxylase in bundle sheath cells of certain C_4-dicarboxylic acid cycle plants which have a low content of malic enzyme.

Recent experiments with isolated cell types from *Digitaria sanguinalis* leaves have established that a major photosynthetic CO_2 fixation pathway is via the β-carboxylation activity of PEP†† carboxylase in the mesophyll cells. The OAA formed is reduced primarily to malate which, in the bundle sheath cells, is decarboxylated by malic enzyme to form pyruvate, CO_2, and NADPH. This CO_2 in the bundle sheath cells then is reduced to the level of carbohydrate via the classical reductive pentose phosphate cycle (1-3). A similar series of reactions have been tentatively proposed for photosynthesis in other plants such as sugarcane and corn (4).

In the current literature these plants are referred to as C_4 plants and they are characterized by the primary fixations of CO_2 into OAA, malate, and aspartate in short time (under 10 sec.) photosynthesis in contrast to pentose plants in which PGA is the first compound detected in short time photosynthesis.

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††Abbreviations used are: PEP, phosphoenolpyruvate; C_4, C_4-dicarboxylic acid; pentose, reductive pentose phosphate cycle; chl, chlorophyll; PGA, 3-phosphoglyceric acid; OAA; oxaloacetic acid; and DTT, dithiothreitol.

Malic enzyme is required for the C_4 cycle to operate as postulated. Recently however, the level of malic enzyme has been reported to be 10 to 40 times lower than the rate of leaf photosynthesis in certain C_4 plants including *Atriplex rosea*, *A. nummularia*, *A. spongiosa*, *Chloris gayana*, *Amaranthus edulis*, *Eragrostis brownii*, *Panicum maximum*, *P. texanum*, and *Cynodon dactylon* (5-8). We have searched for alternate reactions for the release of CO_2 in the bundle sheath cells of these plants and this report presents data to show that PEP carboxykinase can fulfill this role in certain C_4 plants.

The PEP carboxykinase reaction first was demonstrated clearly by Utter and Kurahashi (9) in liver and it has been detected in leaf extracts of several pentose plants (10). However, PEP carboxykinase was not detectable in leaf extracts from C_4 plants such as sugarcane, corn, and sorghum (11). The reversible reaction catalyzed by the plant enzyme is shown below (10).

$$OAA + ATP \longleftrightarrow PEP + CO_2 + ADP \qquad (1)$$

METHODS

Most of the plants listed in Table III were grown in the greenhouse at a light intensity of 3000 to 4000 foot candles. However some plants including *Digitaria sanguinalis*, *Cynodon dactylon*, *Amaranthus retroflexus* and *Sparobolus poiretii* were vigorously growing field specimens harvested in early summer in Athens, Georgia. Segments (2-3 mm) of mature leaves were ground in a mortar with 10 ml of grinding medium. Unless otherwise specified the grinding medium consisted of 50 mM HEPES buffer, pH 6.8, 5 mM DTT, 2 mM EDTA, 5 mM $MgCl_2$, and 5 mM $MnCl_2$. The mortar ground homogenate was shaken for 30 seconds with a Braun Cell Homogenizer which resulted in complete breakage of cells. The homogenate was filtered through a 20 micron nylon net to remove cellular debris. Enzyme assays were run on the homogenate or where specified the homogenate was centrifuged at 25,000 x g for 15 min.

PEP carboxykinase was assayed by the exchange reaction as described by Mazelis and Vennesland (10). The reaction mixture included 10 mM OAA, 6 mM

$NaH^{14}CO_3$, 5 mM ATP, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM DTT, 50 mM HEPES buffer, pH 6.8, and various concentrations of enzyme in a total volume of 0.15 ml.

PEP carboxykinase also was assayed by following the carboxylation reaction directly:

$$PEP + {}^{14}CO_2 + ADP \longleftrightarrow {}^{14}C\text{-}OAA + ATP \qquad (2)$$

The reaction mixture included 10 mM PEP, 6 mM $NaH^{14}CO_3$, 5 mM ADP, 5 mM $MnCl_2$, 5 mM DTT, 50 mM MES buffer, pH 6.0, and enzyme in a total volume of 0.15 ml. The PEP carboxylase activity at pH 6.0 was determined in a similar assay in the absence of ADP (8). Malic enzyme and the NADH dependent malic dehydrogenase were assayed by routine procedures previously described (1,8). To identify the labeled compound(s) in the exchange reaction the reaction was killed with dinitrophenylhydrazine. The hydrazones were extracted by standard procedures and chromatogramed on Whatman No. 4 paper in butanol-ethanol-0.5 M NH_4OH (7:1:2, v/v) and identified by comparisons with authentic compounds.

RESULTS AND DISCUSSION

In experiments designed to determine if OAA or aspartate could be used photosynthetically by bundle sheath cells of C_4 plants which had low levels of malic enzyme (8), we found with crude extracts of *Panicum maximum* leaves a

TABLE I

PEP CARBOXYKINASE ACTIVITY IN *PANICUM MAXIMUM*

Experiment No.	Reaction Components	Activity
		µmoles/mg chl/hr
1	OAA + ATP	576
1	pyruvate + ATP	1
1	OAA	1
2	OAA + ATP	630
2	OAA + ADP	505
2	OAA + GTP	320
2	OAA + ITP	280

The reaction mixtures contained 10 mM OAA or pyruvate and 5 mM of the various nucleotides. Other components were as described in Methods for the exchange reaction assay.

large stimulation of $^{14}CO_2$ incorporation when OAA plus ATP was used. Since many other enzymes in leaves are active with the substrates in reactions (1) and (2) it was essential first to characterize the reaction to be sure of the identity. The PEP carboxykinase from *P. maximum* has been studied more extensively than the enzyme from other plants; so much of the results will be with *P. maximum*. In Table I some requirements of the exchange reaction are given for leaf extracts from *P. maximum*. The exchange reaction has an absolute requirement for both OAA and a nucleotide. In confirmation of previous work with the plant enzyme (10) the adenosine nucleotide derivatives are favored.

TABLE II

EFFECT OF pH ON PEP CARBOXYKINASE AND PEP CARBOXYLASE IN *PANICUM MAXIMUM*

Reaction Mixture Components	pH 6.0	pH 8.0
	μmoles/mg chl/hr	
PEP + $NaH^{14}CO_3$	16	271
PEP + $NaH^{14}CO_3$ + ADP	391	739
PEP + $NaH^{14}CO_3$ + phenylhydrazine	14	301
PEP + $NaH^{14}CO_3$ + ADP + phenylhydrazine	536	664

The reactions performed at pH 6.0 contained 50 mM MES-NaOH buffer, and at pH 8.0 contained 50 mM Tris-HCl buffer.

When assayed from the direction of PEP formation, reaction (2), it was necessary to distinguish the PEP carboxykinase activity from PEP carboxylase activity. In Table II it can be seen that the reaction pH could be varied and at pH 6.0, where PEP carboxylase has a low activity, the PEP carboxykinase could be demonstrated. Trapping of the OAA as its phenylhydrazone seemed to stimulate the reactions somewhat but the basic observation is identical whether the OAA is trapped or not. The phenylhydrazones formed in these experiments and in exchange reaction experiments were chromatographed (see Methods) and the radiochromatogram scanner showed a single peak of radioactivity with an Rf identical to the phenylhydrazone derivative of authentic OAA. The pH

TABLE III

OCCURRENCE OF VARIOUS ENZYMES IN LEAVES OF PLANTS WITH DIFFERENT CYCLES OF PHOTOSYNTHETIC CO_2 FIXATION

Plant	Major CO_2 Cycle	Malic Enzyme	PEP Carboxykinase	PEP Carboxylase	Malic Dehydrogenase
			µmoles/mg chlorophyll/hour		
Panicum maximum	C_4	17	576	141	9650
Panicum texanum	C_4	< 1	378	184	4510
Panicum antidotale	C_4	526	38	632	10410
Panicum miliaceum	C_4	32	< 1	160	7550
Panicum bisulcatum	Pentose	< 1	1	8	6760
Sporobolus poiretii	C_4	30	470	2060	1547
Digitaria sanguinalis	C_4	400	19	388	-
Digitaria decumbens	C_4	231	9	438	1151
Saccharum officinarum	C_4	625	< 1	344	-
Amaranthus retroflexus	C_4	120	< 1	1310	-
Andropogon virginicus	C_4	181	7	286	1280
Andropogon scoparius	C_4	162	7	601	1035
Eragrostis curvula	C_4	18	1	622	1310
Cynodon dactylon	C_4	42	1	254	6770
Spinacia oleracea	Pentose	-	4	-	8350

optimum for PEP carboxykinase has been examined in more detail and the pH optimum is approximately 6.8.

The PEP carboxykinase has a definite metal requirement in that Mg^{++} or Mn^{++} must be added. However the details of the metal requirement remain to be fully investigated. Both Mn^{++} and Mg^{++} are added routinely to all assay mixtures. Routinely activity response curves are performed and the exchange reaction in all leaf extracts is linear for at least 5 minutes and is proportional to enzyme concentration. From these results and from comparisons with other published data (9,10), we conclude that the enzyme is PEP carboxykinase.

Once PEP carboxykinase was detected in *P. maximum* we realized that it might function essentially as an OAA decarboxylase to furnish CO_2 for the bundle sheath cells of C_4 plants without sufficient malic enzyme. Numerous plants then were assayed for PEP carboxykinase activity (Table III). Three C_4 species were found with high levels of enzyme: *P. maximum; P. texanum;* and *Sporobolus poiretii*. Low levels of PEP carboxykinase were detectable in leaf extracts from all pentose plants we have examined (unpublished data and Table III) similar to previous work (10). There are some C_4 plants with low levels of malic enzyme which also have low levels of PEP carboxykinase, e.g., *P. miliaceum*, *Eragrostis curvula*, and *Cynodon dactylon*. The generation of CO_2 in the bundle sheath cells of these plants is under investigation.

In order to more fully understand the role of PEP carboxykinase in leaf metabolism we have obtained data on enzyme localization in specific cells. The importance of enzyme localization lies in the fact that leaves of C_4 plants have very distinct photosynthetic cell types (3,4,12) which have definite functions in the C_4 cycle of photosynthesis and in other aspects of leaf metabolism (3). Table IV shows that PEP carboxykinase is localized in the bundle sheath cells. It is noteworthy that in *P. maximum* the enzyme is sufficiently active in mesophyll cells that one could speculate on a direct role in fixation of atmospheric CO_2. From 40 to 50% of the enzyme activity in crude

TABLE IV

LOCALIZATION OF PEP CARBOXYKINASE AND PEP CARBOXYLASE IN LEAF CELL TYPES

Plant	Enzyme	Mesophyll Cells	Bundle Sheath Cells
		μmoles/mg chl/hr	
Panicum maximum	PEP carboxykinase EXP 1	186	752
	EXP 2	131	553
	PEP carboxylase	251	9
Digitaria decumbens	PEP carboxykinase	2	76
	PEP carboxylase	370	58
Digitaria sanguinalis	PEP carboxykinase	1	57
	PEP carboxylase	348	148

extracts of bundle sheath cells of *P. maximum* can be recovered in a particulate fraction containing chloroplasts and mitochondria. PEP carboxykinase in some animal tissues has been localized in mitochondria and prominent mitochondria are present in bundle sheath cells of most C_4 plants (4,12). We can not at the moment state whether the C_4 plant enzyme is a chloroplast or mitochondrial enzyme but it does appear in pellets containing these organelles. The exact role of PEP carboxykinase is uncertain in leaf metabolism but we favor it acting primarily as a decarboxylase in the bundle sheath cells of C_4 plants, furnishing CO_2 as a substrate for the pentose cycle.

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