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Histochemical Approach to Properties of *Vicia faba* Guard Cell Phosphoenolpyruvate Carboxylase[1]

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WILLIAM H. OUTLAW, JR., JILL MANCHESTER, AND CYNTHIA A. DICAMELLI
Department of Biology (1137), Washington University, Saint Louis, Missouri 63130

ABSTRACT

Properties of phosphoenolpyruvate carboxylase in guard cells dissected from frozen-dried *Vicia faba* L. leaflets were studied using quantitative histochemical techniques. Control experiments with palisade cells and whole leaflet extract proved that the single cell approach was valid. Most characteristics of enzyme activity in guard cells were identical to those in the leaflet extract. The activities were highly dependent on temperature, with maximum activity at 25 to 35 C. Half-maximum activity (with 1 millimolar phosphoenolpyruvate [PEP]) was observed at 0.1 millimolar Mg^{2+}. Two-hundred millimolar NaCl inhibited the reaction by 50%. With frozen-dried leaflet extract, the apparent $K_{m(PEP)}$ was 0.15 millimolar at pH 7.7; with guard cells, the values were 1.49, 0.5 to 0.8, and 0.24 millimolar in three successive experiments. Additional experiments showed that apparent $K_{m(PEP)}$ of guard cell activity from plants within a single growth lot was reproducible and did not change during stomatal opening. Mixed extract experiments proved that soluble compounds were not responsible for the difference observed between leaflet and guard cell activities. The differences in apparent $K_{m(PEP)}$ of guard cell activity could not be unambiguously interpreted. The physiological implications of the properties of this enzyme in guard cells are discussed.

The basis for swelling of guard cells as stomates open is the large negative osmotic potential resulting from K ion uptake. Guard cell K concentration changes several hundred millimolar during opening (2, 10, 12, 13). Some of the K ion uptake is electrically balanced by chloride influx (12, 14, 15) but the bulk of it is balanced by release of protons during malate and citrate synthesis (10). Thus, anion synthesis in guard cells plays a key role in stomatal functioning. In particular, the anaplerotic step, which is catalyzed by PEP[2] carboxylase (9), is a metabolic branch point and is likely to be highly regulated. In this paper, we report some of the properties of guard cell PEP carboxylase of *Vicia faba* L.

MATERIALS AND METHODS

Plant Material. *V. faba* L. was used in all experiments. Plant materials for most macroexperiments were glasshouse-grown; in some cases, plants were grown under a mixture of fluorescent and incandescent lights. Plant material for most microexperiments was cultured as described earlier (*i.e.* sunlight supplemented by artificial light, ref. 3). The histochemical data reported in Figures 1 and 2 and experiment 2 of Figure 4 were from the open leaflet reported as experiment 1 in earlier studies (10). The palisade cells (Fig. 3) and guard cells (experiment 1 of Fig. 4) were from the leaflet used for previous PEP and malate analyses (9). Leaflets for Figure 5 were also from this same growth lot. The leaflet for experiment 3 of Figure 4 was grown under two 20-w fluorescent tubes and two 150-w incandescent bulbs.

Extraction and Assay of PEP Carboxylase. Procedures for extraction and assay of PEP carboxylase (Pi: OAA carboxylase [phosphorylating], EC 4.1.1.31) have been published in detail (9). Typically, a macroassay was initiated by adding tissue extract to 1 ml of reagent. OAA formation was followed in a malate dehydrogenase coupled system. Microanalysis was usually initiated by adding 5 to 15 ng (dry weight) of tissue to 1 μl of reagent ("oil well technique," 6). OAA formed was linked to the oxidation of NADH. NAD^+ was enzymically amplified (5). Exceptions to these procedures are noted in the text. Because of the extreme sensitivity of the enzyme to pH, most assays were performed with a single buffer stock. All apparent K_m values for PEP were measured at pH 7.7.

Wilkinson's method (18) for statistical analysis of enzyme kinetics was used.

Mixed Extract Experiments. One guard cell pair (about 6 ng [dry weight]) was added to 0.5 μl of Tris-HCl (pH 7.7) in the oil well. Endogenous enzyme activity was heat-inactivated (70 C, 30 min). PEP was added in 0.1 μl. The reaction was initiated by addition of double strength assay reagent (except no PEP) containing frozen-dried leaflet extract. In the reverse experiment, frozen-dried leaflet extract was diluted to 20 ng (dry weight)/μl of Tris-HCl (pH 7.7). Endogenous enzyme activity was heat-inactivated (75 C, 30 min). Five-tenths μl was delivered into oil wells and 0.5 μl of double-strength reagent was added ($[PEP]_{final}$ = 1.4 mM). The reaction was initiated by the addition of a guard cell pair. (Guard cells were from the leaflet used for experiment 2 of Fig. 4).

Ammonium Sulfate Fractionation. Extracts were diluted to 0.85 mg protein/ml. (Protein was estimated spectrophotometrically [4].) Crystalline $(NH_4)_2SO_4$ (Schwarz/Mann) was added to the extract which was maintained at 0 to 4 C during the entire procedure. After 20 min, the sample was centrifuged. The supernatant was removed and additional $(NH_4)_2SO_4$ was added to it. This procedure was repeated until about nine fractions between 1 and 4 M $(NH_4)_2SO_4$ were collected. The precipitated protein in each fraction was dissolved in 100 mM Tris-HCl (pH 8.1) containing 1 mM $MgCl_2$. Samples were desalted by passage through a Sephadex G-25 (Pharmacia) column (1 × 10 cm) which had been equilibrated with the above buffer. Fractions were assayed for PEP carboxylase activity (pH 7.7 and 2 mM PEP) and apparent $K_{m(PEP)}$.

Column Chromatography. Extracts were concentrated by precipitation with 4 M $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation and redissolved in 200 mM Na-phosphate (pH 6.5) containing 0.5 mM DTT, 0.5 mM EDTA, and 0.1 mM PEP. The preparation was passed through a Sephadex G-200

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[2] Abbreviations: PEP: phosphoenolpyruvate; OAA: oxaloacetate.

(Pharmacia) column (1.6 × 30 cm) which had been equilibrated with the above buffer solution. One-ml fractions were collected and assayed for activity (pH 7.7 and 2 mM PEP).

RESULTS

Effect of Temperature. Product accumulation by PEP carboxylase in guard cells and palisade cells was four to five times greater at 25 C than at either 10 or 40 C (Fig. 1). The approximate time course for the reaction using fresh leaflet extract indicated enzyme instability above 35 C (not shown). The initial rate of the reaction with fresh leaflet extract was about 35% greater at 40 than 25 C (not shown). There was no difference among the data for whole leaflet, palisade cells, or guard cells.

Cation Requirement and Salt Inhibition. PEP carboxylase activity from fresh leaflets, frozen-dried leaflets, palisade cells, and guard cells showed a strong and similar dependency on Mg^{2+} (Fig. 2). When no divalent cation was added, the activity was about 10% of maximum activity. Half-maximum activity was at 0.1 mM Mg^{2+}. With fresh leaflet extract, Mg^{2+} could be replaced by Mn^{2+}.

Two hundred mM NaCl (or K-acetate, not shown) inhibited PEP carboxylase activity in guard cells and palisade cells to the same extent (Fig. 2).

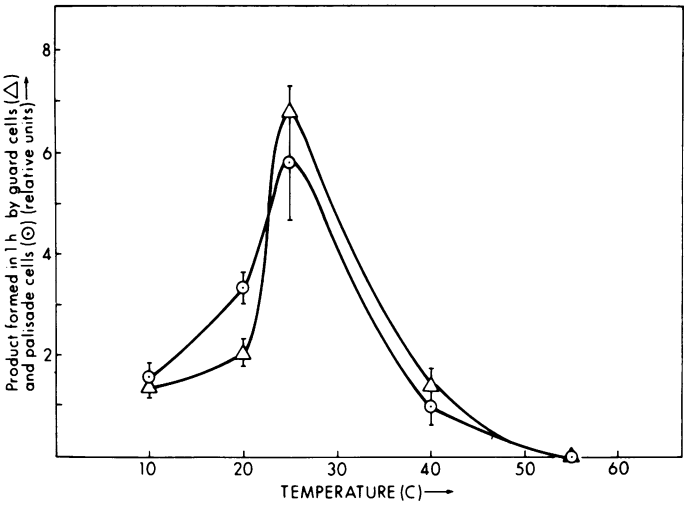

FIG. 1. Effect of temperature on product formed in 1 h by PEP carboxylase in guard cells (△) and palisade cells (⊙) dissected from frozen-dried leaflet.

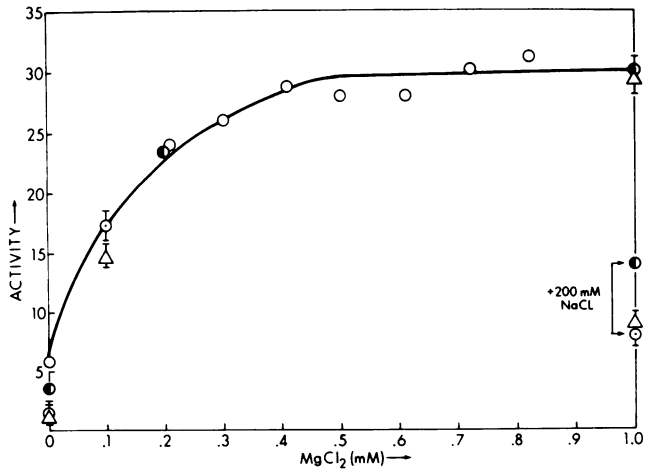

FIG. 2. Dependence of PEP carboxylase activity on Mg^{2+} with extract of whole leaflet (○), frozen-dried leaflet (◐), guard cells (△), and palisade cells (⊙). Inhibition of the reaction by 200 mM NaCl is shown for frozen-dried leaflet, guard cells, and palisade cells at 1 mM $MgCl_2$.

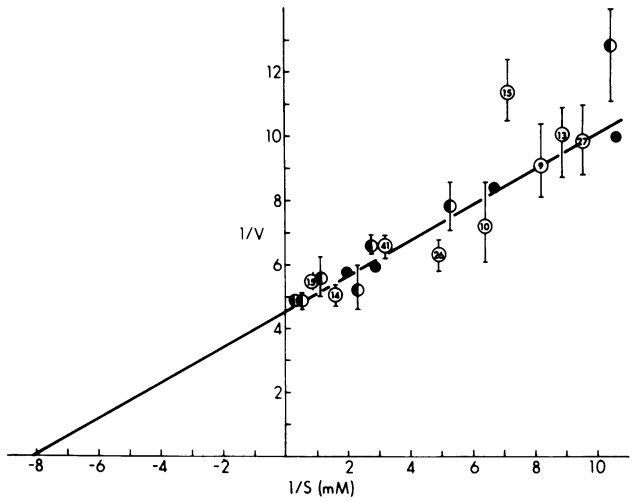

FIG. 3. Apparent $K_{m(PEP)}$ of PEP carboxylase activity in various extracts of frozen-dried tissue. Assay pH was 7.7 and Mg^{2+} was 1 mM. Statistical analysis of the data showed the K_m to be 0.156 ± 0.027 mM for frozen-dried leaflet extract (◐), 0.120 ± 0.016 mM for whole leaflet extract in the presence of previously heated guard cell extract (●), and 0.123 ± 0.029 mM for palisade parenchyma cells (⊗, number of samples at each point shown inside the circle). SE indicated by vertical bar.

PEP Saturation Kinetics with Leaflet, Palisade Cell, and Root Enzyme. The apparent $K_{m(PEP)}$ of PEP carboxylase (24 C, 1 mM $MgCl_2$ [pH 7.7], 0.5 mM $NaHCO_3$ in all experiments) was 0.4 to 0.5 mM when the enzyme was extracted from fresh leaflets (reported earlier [9] and data not shown). The apparent $K_{m(PEP)}$ of the fresh leaflet did not change for at least 6 h after extraction. If the leaflets were previously quick-frozen and frozen-dried, the apparent $K_{m(PEP)}$ was 0.156 ± 0.027 mM (Fig. 3), which was similar to that of palisade cells dissected from frozen-dried leaflets (0.123 ± 0.029 mM, Fig. 3). In other experiments, the enzyme in fresh leaflet extract was precipitated in 4 M $(NH_4)_2SO_4$ and redissolved; the apparent $K_{m(PEP)}$ was 0.15 mM. When the extraction buffer included either 5% (w/v) BSA or 2 M KCl, the apparent $K_{m(PEP)}$ in fresh leaflet extract was 0.152 mM and 0.164 mM, respectively. (High concentration of KCl resulted in substantial loss of enzyme activity.) Inclusion of 1 mM DTT in the extraction buffer for frozen-dried leaflet did not cause a change in apparent $K_{m(PEP)}$ (not shown).

The apparent $K_{m(PEP)}$ of PEP carboxylase extracted from fresh root was 0.2 mM; the PEP concentration for half-maximum velocity with extract from frozen-dried roots was 0.2 mM (nonlinear Lineweaver-Burk plot, but only one experiment.

The apparent $K_{m(PEP)}$ of PEP carboxylase extracted from frozen-dried leaflets was 0.120 ± 0.016 mM (Fig. 3) in the presence of heat-inactivated guard cell extract.

PEP Saturation Kinetics with Guard Cell Enzyme. The apparent $K_{m(PEP)}$ of the guard cell enzyme at pH 7.7 was determined in three experiments. In experiment 1 (same leaflet as palisade cells, Fig. 3), the apparent $K_{m(PEP)}$ was 1.49 ± 0.37 mM (Fig. 4). In experiment 2 (Fig. 4), the apparent $K_{m(PEP)}$ was 0.5 mM if the data points were fitted by linear regression. However, the data did not fit the Michaelis-Menten curve well (points for lowest PEP concentrations not shown). The data from the lowest substrate concentration extrapolated to an apparent $K_{m(PEP)}$ of 0.8 mM. In experiment 3, the apparent $K_{m(PEP)}$ of guard cell enzyme was 0.24 mM which was slightly less than twice that of the activity from frozen-dried leaflets. The differences among the experiments of the apparent $K_{m(PEP)}$ (Fig. 4) were not due to product accumulation by PEP carboxylase (≤10 μM). Also, a test showed that [PEP] did not change significantly during the assay.

Guard cell enzyme velocity assayed at a limiting PEP concentration was not affected by the presence of heat-inactivated leaf extract (data not shown).

An experiment was performed to test the possibility that the different results shown in Figure 4 were due to a reversible protein modification. Guard cells were dissected from leaflets which were at various stages of stomatal opening and the enzyme activities at two different limiting substrate concentrations were compared (Fig. 5). The upper hatched region is the range of ratios calculated for the apparent $K_{m(PEP)}$ ± SE of palisade cell enzyme. The lower hatched region is the range of ratios calculated for the apparent $K_{m(PEP)}$ ± SE of the guard cell enzyme (experiment 1 of Fig. 4). Only one of the ratios falls outside the region delineated by the data for guard cell enzyme. For convenience in interpretation, the rate of malate accumulation in these guard cells *in vivo* (*i.e.* immediately before freezing) is plotted from reference 9.

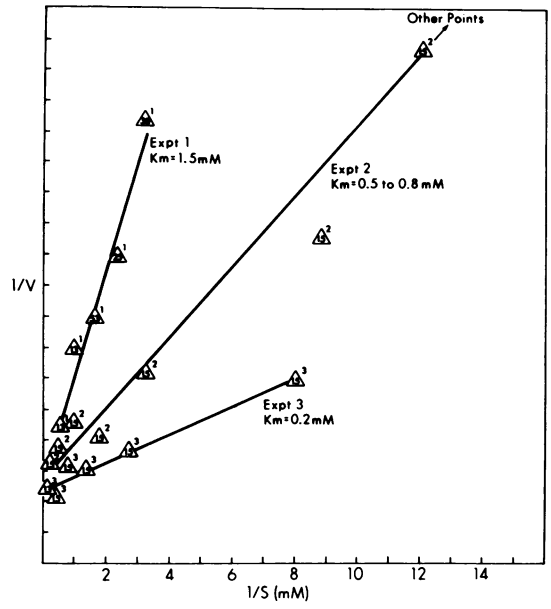

FIG. 4. Lineweaver-Burk plot of PEP carboxylase activity in frozen-dried guard cells *versus* PEP. Assay pH was 7.7; Mg^{2+} and HCO_3^- concentrations were 1 mM and 0.5 mM, respectively. Number inside triangle shows number of assays at that point. Superscript by symbol designates experiment. SE of activities averaged 6% in all three experiments. (Experiment 1 leaflet was also the source for palisade cells [Fig. 3] and in the same growth lot as the leaflets used for Fig. 5.)

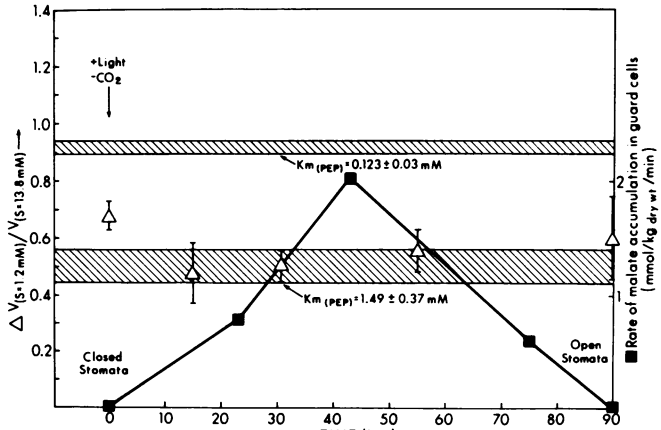

FIG. 5. Ratio of guard cell PEP carboxylase activity at pH 7.7 and 1 mM Mg^{2+} with 1.2 mM PEP and 13.8 mM PEP (△). Guard cells were dissected from frozen-dried leaflets which were harvested at various times during stomatal opening. Apparent $K_{m\,(PEP)}$ of guard cell enzyme was 1.49 ± 0.37 mM (N = 128) at 31 min after stomatal opening was induced [$V_{(S=1.2mM)}/V_{(S=13.8mM)}$ shown by the lower hatched bar (see also experiment 1 of Fig. 4)]; apparent $K_{m(PEP)}$ of palisade cell enzyme was 0.123 ± 0.03 mM from same leaflet [$V_{(S=1.2mM)}/V_{(S=13.8mM)}$ shown by upper hatched bar (see also Fig. 3)]. Rate of malate accumulation in guard cells *in vivo* is shown by closed squares (replotted from ref. 9).

Column Chromatography and Ammonium Sulfate Precipitation. Epidermal peel and leaflet PEP carboxylase activities eluted coincidentally from a G-200 column and showed the same solubility in $(NH_4)_2SO_4$ (data not shown). The apparent K_m values for PEP of fractions from epidermal peel extract were between 0.139 and 0.152 mM.

DISCUSSION

Palisade parenchyma is one of the most common cell types in *Vicia* leaflet. This cell type served as a control to evaluate the comparability of the microassay and the conventional method of extraction, dilution and analysis in ml volumes. The microtechnique differs in several respects; *e.g.* the individual cell wall is often intact during analysis (11). Thus, the wall acts as a tiny dialysis bag through which substrates and products are presumed to diffuse. As a result, potentially interfering (endogenous) enzyme activities are sequestered in the environment of product formation. Another difference is that the frozen-dried sample is immersed in oil before hydration in the assay solution. Nevertheless, all of the investigated properties of the palisade cell PEP carboxylase (microassay) were similar to frozen-dried leaflet PEP carboxylase (conventional technique). We concluded that the single cell approach to studying the properties of PEP carboxylase is valid.

The pronounced decrease in guard cell PEP carboxylase activity at higher temperature (Fig. 1) may partially explain the rapid decrease in stomatal aperture in *Vicia* above 35 C (17). The effects of temperature on stomatal opening are complex, of course, and would not be explained by the response of this one enzyme alone.

The Mg^{2+} requirement for whole leaflet PEP carboxylase approximated normal Michaelis-Menten kinetics in the range of 0.1 to 1 mM when assayed at pH 8.7 with 1 mM PEP (Fig. 2). The main value of these data is for comparative purposes because some Mg^{2+} was carried over into the assay solution from the crude extract and a narrow range of cation concentrations was tested. Nevertheless, some interpretation can be made in view of the following: the substrate of PEP carboxylase is PEP-Mg^{2+} (7, 8, 16). The [PEP-Mg^{2+}] was about 16, 62, and 143 μM for 0.1, 0.4, and 1 mM Mg^{2+}_{total}, respectively (K_d values of 4.76×10^{-3} and 5.56×10^{-3} are published in refs. 16 and 19, respectively). Simultaneous solution of Michaelis-Menten equation for 0.4 mM and 0.1 mM Mg^{2+}_{total} yielded a $K_{m(PEP\text{-}Mg^{2+})}$ of 18 μM [(pH 8.7), ignoring effect of Mg^{2+}_{free} and PEP_{free}]. Over the range from 0.4 mM to 1 mM Mg^{2+}_{total}, the $[Mg^{2+}]_{free}$ increased from 338 to 857 μM (= 0.5 mM). PEP_{total} was 17 times the apparent $K_{m(PEP)}$ at 1 mM Mg^{2+}_{total} and $[PEP]_{free}$ decreased only 9%. The reaction rate (Fig. 2) was unchanged. These results are consistent with the earlier findings that the reaction was not very sensitive to variation in the level of Mg^{2+}_{free} if $[Mg^{2+}]_{free}$ was low (7, 8).

The role of Mg^{2+} in the regulation of guard cell PEP carboxylase at low [PEP] can not be ignored because the apparent $K_{m(PEP)}$ of guard cell PEP carboxylase [Fig. 4 (pH 7.7, Mg^{2+} = 1 mM)] exceeds guard cell [PEP] (9). It is calculated that increasing Mg^{2+} from zero to infinity lowers the $K_{m(PEP)}$ from 1,000 to 108 μM at pH 7.5 (spinach leaf, 7). In another report (maize leaf, 8), the $K_{m(PEP\text{-}Mg^{2+})}$ was decreased from 21 to 2 μM (pH 8.5) when $[Mg^{2+}]_{free}$ was decreased from 2.3 to 0.1 mM. Also, guard cell [Mg^{2+}] will fluctuate during stomatal opening unless there is a net exchange with the surroundings. Lacking estimations of guard cell [Mg^{2+}], further evaluation of the role of this cation in the regulation of anion synthesis is premature.

PEP carboxylase activity in *Vicia* leaflets exists in a low apparent $K_{m(PEP)}$ form (about 0.15 mM) and a high apparent $K_{m(PEP)}$ form (about 0.45 mM) when assayed at pH 7.7 and Mg^{2+} = 1 mM. The high apparent $K_{m(PEP)}$ form was observed if fresh leaflets were

extracted conventionally. The low apparent $K_{m(PEP)}$ form was first found in extracts of frozen-dried leaflet. High protein concentration and high ionic strength are characteristics of freeze-drying. Extraction of fresh leaflet in buffer with high protein or high ionic strength resulted in the low apparent $K_{m(PEP)}$ form. In fact, precipitation with $(NH_4)_2SO_4$ converted the high apparent $K_{m(PEP)}$ form to low apparent $K_{m(PEP)}$ form. It is probable that the low K_m form is the *in vivo* state.

The apparent $K_{m(PEP)}$ (pH 7.7, 1 mM Mg^{2+}) of guard cell PEP carboxylase activity was different in each of three experiments (Fig. 4). The differences were not found from leaf to leaf within one growth lot but were evident among the growth lots. These differences were probably not owing to assay interference by soluble contents of guard cells because of the 100,000× dilution of cell volume during the assay and the fact that the mixing experiments were negative. It is also unlikely that a reversible protein modification (*e.g.* phosphorylation) could explain the differences because the apparent $K_{m(PEP)}$ was the same regardless of the rate of malate synthesis in the guard cells which were subsequently used in the *in vitro* assay (Fig. 5). Several explanations (*e.g.* different levels of guard cell specific isoenzyme, activation, etc.), could be proposed, but presently, no unambiguous interpretation can be made.

The apparent $K_{m(PEP)}$ of guard cell PEP carboxylase was higher at pH 7.7 (Fig. 4) than at pH 8.7 (9). We suggest that this pH-stat mechanism (see ref. 1) makes organic anion synthesis in guard cells directly responsive to cytoplasmic alkalinization during proton efflux.

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