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Lessened malate inhibition of guard-cell phospho*enol*pyruvate carboxylase velocity during stomatal opening

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Abstract Leaflets of *Vicia faba* with closed stomata or with opening stomata were freeze-dried. Excised guard-cell pairs were assayed individually under suboptimal conditions (pH 7.1 and subsaturating substrate) for phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) using quantitative histochemical procedures. L-Malate, 400 μM, significantly inhibited guard-cell PEPC activity of closed stomata but not that of opening stomata. We postulate that the lessened sensitivity of guard-cell PEPC activity to malate inhibition is an important regulatory feature of stomatal opening, which is associated with malate accumulation.

Key words: Guard cell; L-Malate; Phospho*enol*pyruvate carboxylase; Stoma; *Vicia faba*

1. Introduction

Uptake of atmospheric CO_2 into leaves occurs through stomata in the epidermis. These microscopic pores are nonselective with respect to gasses and must be regulated to prevent excessive water-vapor loss from the leaf-intercellular spaces. Each stoma is the pore between two elongated guard cells that lie parallel. The aperture size is increased when guard cells take up water and swell, because their cell walls distend asymmetrically [1]. (Conversely, when guard cells lose water, the pore narrows.) Water influx is an osmotic phenomenon. In brief, proton extrusion by the guard-cell plasmalemma ATPase hyperpolarizes the membrane. Hyperpolarization opens voltage-gated K^+-in channels and creates a difference in electrochemical potential sufficient for the influx of K^+, which, in association with anions, is the primary osmoticum [2–5]. A major mechanism of maintaining cytosolic pH during H^+ extrusion is the synthesis and accumulation of malate^{2-}; this process releases two protons for each divalent malate accumulated [6]. The initial dedicated step is the carboxylation of PEP, the product of which, oxaloacetate, is the immediate precursor of malate. This step is catalyzed by cytosolic PEPC (EC 4.1.1.31; [7]).

Many isoforms of PEPC have evolved to fulfill the diverse metabolic roles of this ubiquitous enzyme activity in plants [8–10]. For example, specific isoenzymes catalyze the initial incorporation of atmospheric CO_2 in the C_4 and CAM auxiliary photosynthetic pathways. In these examples, it is well established that PEPC phosphorylation causes a reversible activation of this enzyme [11] to meet temporal physiological demands. Activated thus, PEPC has decreased sensitivity to its negative allosteric effector L-malate. Other mechanisms [12] may also be involved in modulating PEPC activity, and the PEPC velocity is strongly influenced by chemical microenvironment, particularly pH [13]. Like the in vivo fluxes of photosynthetic PEPC's, that catalyzed by guard-cell PEPC corresponds to tissue physiological states. We report that guard-cell PEPC from opening stomata of the C_3-plant *Vicia faba* has lower sensitivity to malate than does guard-cell PEPC from closed stomata. This difference in apparent phosphorylation status is a biochemical basis for regulation of anion synthesis. In addition, this phenomenon may provide a means for studying the signal-transduction pathways that cause stomatal movements.

2. Experimental

2.1. Plant materials and sampling

For the reported guard-cell experiments, *Vicia faba* L. cv. Longpod plants were grown in 1-l pots in Metro-Mix 220 potting medium in a growth chamber (16-h day; 600 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux density, provided by a mixture of incandescent and fluorescent lamps; 25/20°C day/night temperature regime). The third fully expanded bifoliate of 21-day-old plants was used in all experiments. Histochemical samples for exploratory experiments were designated 'I' and were from one growth lot, whereas samples designated 'II' and 'III' were from a second and later growth lot. Leaves with 'Closed Stomata' (Figs. 1 and 2) were harvested one hour before the end of the dark period and were immediately frozen in liquid nitrogen. For the preparation of a sample with open stomata, a node of a predarkened plant was excised under water; then, the leaf was cut off under water, after which the base of the petiole was submerged in water that filled a 50-ml Erlenmeyer flask. Up to four such flasks were set into a stainless-steel food-service tray (30 cm × 25 cm × 15 cm (high)) filled to a depth of 1 cm with 0.5 N NaOH. After the laminae were positioned horizontally, the top of the tray was covered with glass and the entire apparatus was transferred to the illuminated growth cabinet, the lights of which were shielded with a heat barrier. Thus, two stimuli (viz., light and low [CO_2] resulting from CO_2 absorption by the NaOH solution) that cause stomatal opening were simultaneously and continuously applied. After 20 min (cf. [14,15]), the leaves with 'Opening Stomata' (Figs. 1 and 2) were harvested and were immediately frozen in liquid nitrogen slurry. (The rate of malate accumulation in guard cells is maximal 20–30 min after closed stomata have been given the described stimuli to open [16].)

2.2. Preliminary PEPC assays with whole-leaf extract

Fresh or frozen leaflets were extracted, 0.3 g leaflet $\cdot ml^{-1}$ extract cocktail, by homogenization at 0°C in 100 mM Tris-HCl (pH 7.1) that contained 1 mM EDTA, 5 mM $MgCl_2$, 0.02% (w/v) bovine serum albumin, and 8 mM dithiothreitol. An extract supernatant fraction was

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Abbreviations: CAM, crassulacean acid metabolism; PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase.

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prepared by a 0.5- to 1-min centrifugation (ca. 13,600 × g, 'Microfuge,' Fisher Scientific). Various treatments to minimize artifacts of proteolysis (separate and combined inclusions of 5 $\mu g \cdot ml^{-1}$ antipain, 0.1 mM benzamidine, 10 $\mu g \cdot ml^{-1}$ chymostatin, 5 $\mu g \cdot ml^{-1}$ leupeptin, or 1 mM phenylmethylsulfonyl fluoride), of changes in phosphorylation status (inclusion of 50 nM okadaic acid), and of low-M_r compounds (passage over Sephadex G-25, 0.2 ml extract · ml^{-1} gel) were tested. Under our assay conditions, rapid extraction and assay, there was no reproducible alteration of sensitivity to L-malate that could be attributed to assay- or extraction-cocktail methods regardless whether the extracts were from illuminated or predarkened leaflet. Attempts to optimize other assay parameters (e.g. PEP concentration, pH) for malate sensitivity were not made; therefore, the main value of these macro experiments was to establish the relevant L-malate concentration range. Depending on the treatment, the total time that elapsed from the initiation of homogenization to the conclusion of the assay was 4 to 6 min.

The PEPC macroassay was initiated by the addition of 7 μl of extract to 1 ml of assay cocktail (identical to the cocktail used for the micro version (Section 2.3) except that NADH concentration was 10 μM, and the L-malate concentration range was 50 to 400 μM). NADH oxidation over 2 to 4 min was measured fluorometrically at 25°C. Wang et al. [17] report other details of this macro version of the PEPC assay.

2.3. *Quantitative histochemical assays of PEPC activity in individual guard-cell pairs*

Fragments, 1 to 3 mm on a side, of frozen leaflet were freeze-dried at −40°C and nominally 10 μmHg for 3 days in a custom-fabricated unit. In a room with controlled temperature and humidity, individual guard-cell pairs were manually dissected from the dried fragments. (Details are given by Passonneau and Lowry [18], and dissection precision is documented by Hampp and Outlaw [19]. For reference, a guard-cell pair has a dry mass of 6 ng [20] and a protein content of 0.3 ng [21].)

The micro PEPC assay was initiated by addition of a guard-cell pair to 16.8 nl of assay cocktail (100 mM Tris-HCl (pH 7.1), 1 mM EDTA, 5.4 mM $MgCl_2$, 0.02% (w/v) bovine serum albumin, 3 mM dithiothreitol, 20 $\mu g \cdot ml^{-1}$ porcine-heart malate dehydrogenase (EC 1.1.1.37), 5 mM $NaHCO_3$, 30 μM NADH (enzymically standardized), ± 0.8 mM PEP (enzymically standardized), and ± 200 or 400 μM L-malate). (We calculate the PEP·Mg concentration in the assay cocktail to be 0.38 mM, or approximately one-half the K_m (see [15]). NADH oxidation was measured fluorometrically at 25°C with a method having a time resolution of seconds and a sensitivity of fmol [22]. (A representative time course is illustrated in [14].) The assay conditions (pH 7.1 and suboptimal PEP concentration in the presence of subsaturating malate) were chosen intentionally to increase the likelihood of detecting a change in malate sensitivity (i.e. apparent phosphorylation status) that would correspond with the physiological state of the tissue (see comments in [11,23]). Endogenous malate [16] contributed an estimated 14 μM ('Closed Stomata') or 20 μM ('Opening Stomata') 'background' malate concentration to all assay cocktails. For Fig. 1, the data from several experiments were consolidated by a program described by Outlaw et al. [24]. Without removal of 'outliers,' the data were analyzed by STATVIEW (Fig. 2). (The results were also analyzed by systematic exclusion of the high and low specific activities in each experimental set (not shown); this analysis permitted the same conclusion as that shown (Fig. 2), but, of course, the apparent experimental variability was smaller.)

3. Results

3.1. *Malate sensitivity of whole-leaflet PEPC*

Typically, malate sensitivity was somewhat higher for whole-leaflet C_3 PEPC extracted from darkened leaflet than for the activity extracted from illuminated leaflet. Example results (9 different leaflet extracts for each physiological state) for PEPC of leaflets receiving 3 to 7 h illumination were 28 ± 3 (S.E.)% inhibition (100 μM L-malate) or 41 ± 2 (S.E.)% inhibition (200 μM malate). The correlate inhibitions for PEPC of pre-darkened leaflet were, respectively, 43 ± 3 and 57 ± 4%. However, the range of values overlapped at the lower malate concentration under these assay conditions, which, as mentioned (Section 2.2), were not optimized for detection of sensitivity changes.

Conventional whole-leaf extracts of fragments of the six leaflets used for dissection of guard cells were also tested for PEPC-malate sensitivity. PEPC from the three leaflets having 'Opening Stomata' was inhibited 23 ± 1 (S.E.)% and 51 ± 2 (S.E.)% by 200 or 400 μM L-malate. The correlate inhibitions for PEPC from the three leaflets having 'Closed Stomata' were 42 ± 3 and 65 ± 4%, respectively.

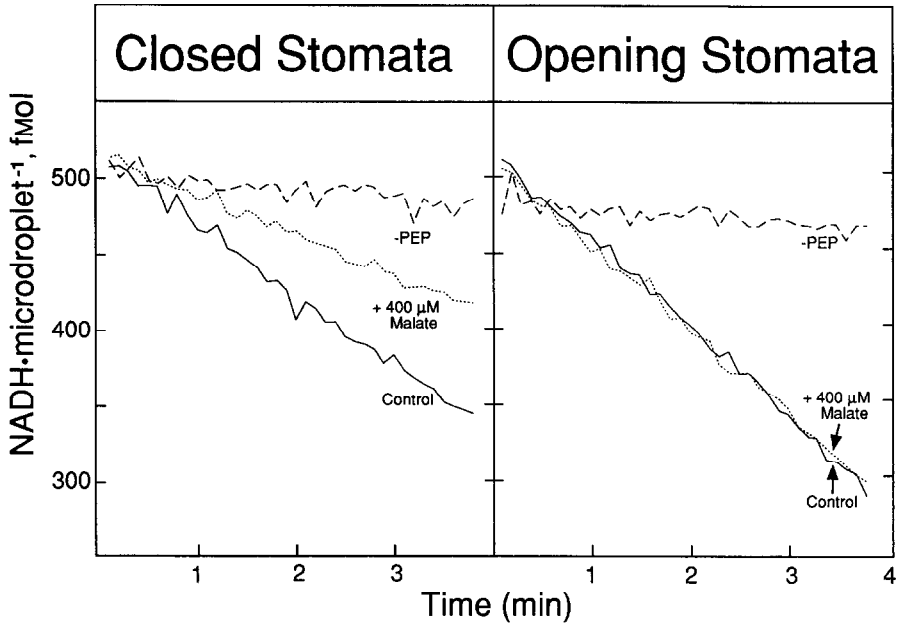

Fig. 1. Time course for the PEP-dependent oxidation of NADH in 16.8 nl of PEPC-assay cocktail in the presence of a guard-cell pair, ± 400 μM L-malate. These traces are the averaged results of the 28 microassays conducted in Experiments II and III (see Fig. 2). The control rate (−PEP) is also shown. Tarczynski and Outlaw [14] show the precision of a single microassay, and Hampp and Outlaw [19] document dissection precision.

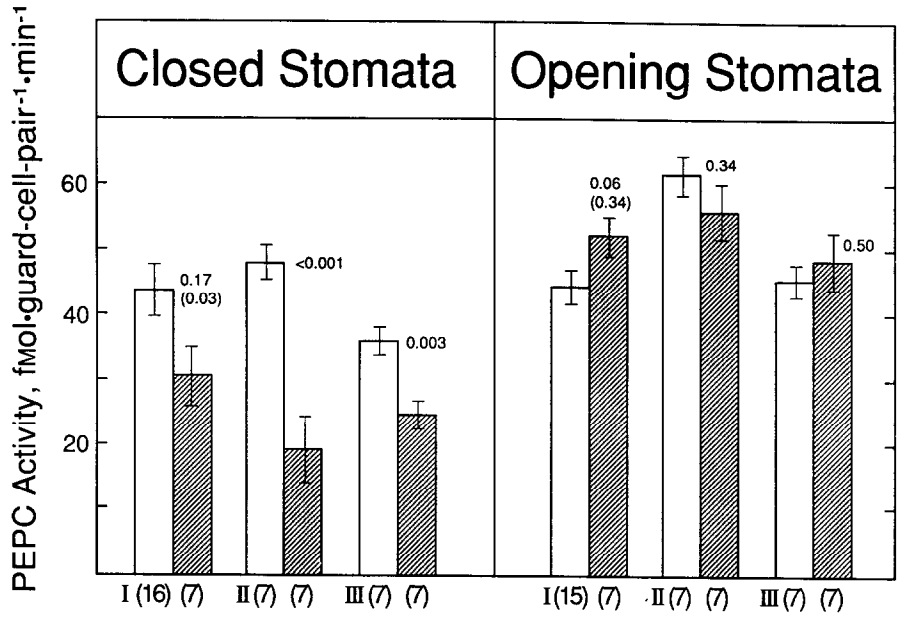

Fig. 2. Efficacy of inhibition by 400 μM L-malate of guard-cell PEPC activity. The open bars are the control rates obtained without malate, and the associated hatched bars are replicate samples assayed in the presence of malate. At the bottom, roman numerals identify the experiment, and arabic numerals in parenthesis indicate the number of individual microassays. The probability, P, that the malate-inhibited velocity is similar to the control velocity is displayed in the chart above the paired bars. (The two values for P in parentheses pertain to tests (not shown) of inhibition by 200 μM L-malate.)

3.2. Malate sensitivity of guard-cell PEPC

Fig. 1 shows the averaged rates of PEP-dependent NADH oxidation by guard-cell 'extracts' of two leaflets having 'Opening Stomata' and of two leaflets having 'Closed Stomata' (i.e. Experiments II and III, which, by design, comprised seven assays for each condition). For all conditions, the time courses were linear, which indicates that PEPC modifications during the assay at least were not revealed kinetically. L-Malate, 400 μM, obviously inhibited guard-cell PEPC of 'Closed Stomata,' but this concentration was without obvious effect on guard-cell PEPC activity of 'Opening Stomata.' t-Tests (Fig. 2) confirmed that neither 200 μM (Experiment I) nor 400 μM (Experiments I–III) L-malate inhibited the activity of guard-cell PEPC of 'Opening Stomata.' In sharp contrast, malate inhibition of guard-cell PEPC of 'Closed Stomata' was highly significant, particularly in Experiments II and III ($P < 0.001$, $P = 0.003$, respectively). Corroboratively, in Experiment I, 200 μM L-malate inhibited guard-cell PEPC activity ($P = 0.03$) of 'Closed Stomata.'

4. Discussion

Activation of the C_4- and CAM-photosynthetic PEPC's (see Section 1) is effected by regulatory phosphorylation, which is manifested kinetically as a decreased sensitivity to malate inhibition when the target enzyme is assayed at a suboptimal pH and PEP concentration [11,23]. As guard-cell malate accumulation is restricted to the period during stomatal opening [16], many researchers have attempted to correlate kinetic alteration of guard-cell PEPCactivity with the physiological state of the tissue. As examples, Kottmeier and Schnabl [25] reported that the K_m(PEP) of guard-cell PEPC is lowered by 20-fold when guard-cell protoplasts are swollen; Donkin and Martin [26] found a 10-fold increase in V_{max} of epidermal-peel PEPC when the tissue is illuminated; Michalke and Schnabl [27] reported 2-min oscillations of guard-cell PEPC activity that occur during K^+-induced protoplast swelling. The preceding results were obtained at assay pH $\geq$ 8, at which Raschke et al. [4] detected neither a V_{max} nor a K_m alteration of guard-cell PEPC that was dependent on the presence of light. However, Raschke et al. [4] did find a 2-fold decrease in K_m when PEPC was extracted from *illuminated* guard-cell protoplasts and assayed at pH 7.0. However, the K_m change was not dependent on external K^+ (K. Raschke, pers. commun.), which is required for stomatal movements. Our previous related studies (e.g. [14,15,17]) also failed to detect an alteration in guard-cell PEPC kinetics corresponding to the physiological state of the tissue, but these studies did not address malate sensitivity of guard-cell PEPC assayed under suboptimal conditions. Here we document that guard-cell PEPC is activated during stomatal opening analogously to the activation of photosynthetic PEPC's during CO_2 uptake. Uncertainties remain, however, as Schnabl et al. [28] reported that there is no light regulation of guard-cell PEPC phosphorylation, which, as discussed, is the molecular correlate of activation of the C_4 and CAM isoforms. Regardless, our findings imply that guard-cell PEPC activity is not solely regulated by changes in the chemical microenvironment of the enzyme as we [14] previously believed.

Notwithstanding the contrary earlier conclusions (which were largely based on assay conditions not appropriate for detection of ΔI_{50} for malate), it now appears that all studied plant PEPC's, not just the photosynthetic isoforms in C_4 and CAM plants, are regulated by reversible protein phosphorylation. C_3-plant PEPC in excised leaves of low-N-grown wheat plants exhibits a light-induced loss of malate sensitivity due to an alteration in the phosphorylation status of the enzyme in

vivo [29,30]. In vitro phosphorylation of soybean-root nodule PEPC by an endogenous proteinkinase causes a loss of sensitivity to L-malate [31]. In a related study, Wang and Chollet [32] purified from the C_3-plant tobacco PEPC that could be phosphorylated in vitro by both homologous and heterologous (i.e. C_4-plant leaf) PEPC kinases. Corroboratively, the C_4-type *Sorghum* PEPC, expressed in *Escherichia coli*, was phosphorylatable, which was accompanied by a reduction in sensitivity to malate [33]. Finally, all reported plant PEPC's (but not those of prokaryotes) contain a highly conserved motif near the N-terminus with a presumptive seryl-phosphorylation site ([34], see also [35]). Our results with guard-cell PEPC, putatively a unique isoform, are corroborative.

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References

[1] Raschke, K. (1979) Encyclopedia of Plant Physiology (New Series) 7, 383–441.
[2] Outlaw, W.H., Jr. (1983) Physiol. Plant. 59, 302–311.
[3] MacRobbie, E.A.C. (1988) Bot. Acta 101, 140–148.
[4] Raschke, K., Hedrich, R., Reckmann, U. and Schroeder, J.I. (1988) Bot. Acta 101, 283–294.
[5] Tallman, G. (1992) Crit. Rev. Plant Sci. 11, 35–57.
[6] Robinson, N. and Preiss, J. (1985) Physiol. Plant. 64, 141–146.
[7] Outlaw, W.H., Jr. (1990) Biochem. Physiol. Pflanz. 186, 317–325.
[8] O'Leary, M.H. (1982) Annu. Rev. Plant Physiol. 33, 297–315.
[9] Latzko, E. and Kelly, G.J. (1983) Physiol. Vég. 21, 805–815.
[10] Andreo, C.S., Gonzalez, D.H. and Iglesias, A.A. (1987) FEBS Lett. 213, 1–8.
[11] Jiao, J. and Chollet, R. (1991) Plant Physiol. 95, 981–985.
[12] Podestá, F.E., Iglesias, A.A. and Andreo, C.S. (1990) Photosynth. Res. 26, 161–170.
[13] Davies, D.D. (1979) Annu. Rev. Plant Physiol. 30, 131–158.
[14] Tarczynski, M.C. and Outlaw, W.H., Jr. (1990) Arch. Biochem. Biophys. 280, 153–158.
[15] Tarczynski, M.C. and Outlaw, W.H., Jr. (1993) Plant Physiol. 103, 1189–1194.
[16] Outlaw, W.H., Jr. and Kennedy, J. (1978) Plant Physiol. 62, 648–652.
[17] Wang, X.C., Outlaw, W.H., Jr., De Bedout, J.A. and Du, Z. (1994) Histochem. J. 26, 152–160.
[18] Passonneau, J.V. and Lowry, O.H. (1993) Enzymatic Analysis: A Practical Guide. Humana Press, Totowa, NJ.
[19] Hampp, R. and Outlaw, W.H., Jr. (1987) Naturwissenschaften 74, 431–438.
[20] Outlaw, W.H., Jr. and Lowry, O.H. (1977) Proc. Natl. Acad. Sci. USA 74, 4434–4438.
[21] Outlaw, W.H., Jr., Manchester, J. and Zenger, V.E. (1981) Histochem. J. 13, 329–336.
[22] Outlaw, W.H., Jr., Springer, S.A. and Tarczynski, M.C. (1985) Plant Physiol. 77, 659–666.
[23] Nimmo, H.G. (1993) in: Society for Experimental Biology Seminar Series 53: Post-translational Modifications in Plants (Battey, N.H., Dickinson, H.G. and Hetherington, A.M., Eds.) pp. 161–170, Cambridge Univ. Press, London/New York.
[24] Outlaw, W.H. Jr., Springer, S.A. and Tarczynski, M.C. (1985) in: Regulation of Carbon Partitioning in Photosynthetic Tissue (Heath, R.L. and Preiss, J., Eds.) pp. 162–179, American Society of Plant Physiologists, Rockville, MD.
[25] Kottmeier, C. and Schnabl, H. (1986) Plant Sci. 43, 213–217.
[26] Donkin, M. and Martin, E.S. (1980) J. Exp. Bot. 31, 357–363.
[27] Michalke, B. and Schnabl, H. (1990) Planta 180, 188–193.
[28] Schnabl, H., Denecke, M. and Schulz, M. (1992) Bot. Acta 105, 367–369.
[29] Van Quy, L., Foyer, C. and Champigny, M.L. (1991) Plant Physiol. 97, 1476-1482.
[30] Van Quy, L. and Champigny, M.L. (1992) Plant Physiol. 99, 344–347.
[31] Schuller, K.A. and Werner, D. (1993) Plant Physiol. 101, 1267–1273.
[32] Wang, Y.H. and Chollet, R. (1993) FEBS Lett. 328, 215–218.
[33] Pacquit, V., Santi, S., Cretin, C., Bui, V.L., Vidal, J. and Gadal, P. (1993) Biochem. Biophys. Res. Commun. 197, 1415–1423.
[34] Toh, H., Kawamura, T. and Izui, K. (1994) Plant Cell Environ. 17, 31–43.
[35] Rajagopalan, A.V., Devi, M.T. and Raghavendra, A.S. (1994) Photosyn. Res. 39, 115–135.