Plant Physiol. (1982) 70, 1582–1585
0032-0889/82/70/1582/04/$00.50/0

Profile of Basic Carbon Pathways in Guard Cells and Other Leaf Cells of *Vicia faba* L.[1]

Received for publication April 20, 1982 and in revised form May 29, 1982

RÜDIGER HAMPP[2], WILLIAM H. OUTLAW, JR., AND MITCHELL C. TARCZYNSKI
Department of Biological Science (Unit I), Florida State University, Tallahassee, Florida 32306

ABSTRACT

Guard cells and three other cell types from *Vicia faba* L. 'Longpod' leaflets were assayed for enzymes that catalyze one step in each of five major carbon pathways in green plants: the photosynthetic carbon reduction pathway (ribulose-bisphosphate carboxylase, EC 4.1.1.39), the photosynthetic carbon oxidation pathway (hydroxypyruvate reductase, EC 1.1.1.81), glycolysis ([NAD] glyceraldehyde-P dehydrogenase, EC 1.2.1.12), the oxidative pentose-P pathway (6-P-gluconate dehydrogenase, EC 1.1.1.44), and the tricarboxylic acid pathway (fumarase, EC 4.2.1.2). Neither ribulose-bisphosphate carboxylase nor hydroxypyruvate reductase could be detected in guard cells or epidermal cells; high levels of these activities were present in mesophyll cells. The specific activity of fumarase (protein basis) was about 4-fold higher in guard cells than in epidermal, palisade parenchyma or spongy parenchyma cells. (NAD) glyceraldehyde-P and 6-P-gluconate dehydrogenases also were present at high protein specific activities in guard cells (2- to 4-fold that in meosphyll cells).

It was concluded that the capacity for metabolite flux through the catabolic pathways is high in guard cells. In addition, other support is provided for the view that photoreduction of CO_2 by these guard cells is absent.

The distinct morphological differences and functions found when guard cells, ordinary epidermal cells, palisade parenchyma cells, and spongy parenchyma cells of C_3 dicotyledon leaves are compared (22) imply biochemical differentiation. Some of these biochemical differences are obvious, *e.g.* Chl is abundant in palisade parenchyma, but is generally lacking in ordinary epidermal cells. Other differences are more subtle; *e.g.* P-enolpyruvate carboxylase is elevated in guard cells (14). Investigators have attempted to determine differences by investigating enzyme distribution among various tissues, but few studies have limited the tissue sample to one particular cell type (*cf.* 14). Even these latter studies have been restricted to enzymes of the photosynthetic carbon reduction pathway and those involved in C_4 acid metabolism. To gain a better understanding of the overall biochemical functioning of these different cells, we have assayed them for enzymes that catalyze steps in major carbon pathways. We report the results in this paper.

MATERIALS AND METHODS

Materials. *Vicia faba* L. 'Longpod' were grown in a growth cabinet (600 μE m^{-2} s^{-1} at plant level, 60% RH; 14-h photoperiod: 25°C/20°C). Fully expanded bifoliate leaves of 3 to 5-week-old plants were used. Analytical enzymes were from Boehringer; most other biochemicals were from Sigma.

Assays. Except for ribulose-bisP carboxylase (19), all assays were optimized with respect to substrate concentrations, buffer, and pH ("Specific Step," Table I) in 1-ml volumes with extracts of *Vicia* mesophyll protoplasts. NAD(P) oxidation/reduction was measured directly by fluorometry. The protoplasts were isolated as before (2) except that 10 mM ascorbate was included in the digestion medium. Extracts were made by rupture of the protoplasts by passage through a 15-μm screen. Chl was determined by the method of Arnon (1).

Pre-illuminated *Vicia* leaflets were quick frozen in melting N_2 and then freeze-dried at −40°C. Extracts of portions of these leaves were assayed for enzyme activity. Except for volume, the "Specific Step" and "First Indicator Step" were as given in Table I. (These controls were designed to verify enzyme activity stability and linearity with time and tissue concentration during steps equivalent to the initial ones used in the histochemistry experiments.) Other portions of these same leaves were used for dissection (40% RH, 19°C). Individual guard cell pairs, palisade parenchymal cells, spongy parenchyma cells, and pieces of epidermis (devoid of guard cells) were weighed on a quartz fiber balance (<20-ng samples). Purity of these samples has been documented earlier (4, 16). The assays were initiated by pushing the sample onto a "Specific Step" reagent droplet (Table I) using the oil well technique (9). In two assays (for ribulose-bisP carboxylase and fumarase), a "First Indicator Step" was required also. Subsequent steps were designed to measures NAD^+ or NADPH by enzymic cycling (5, 7, respectively). Enzymic activities, calculated on a dry weight basis, were converted to a protein basis (20) or Chl basis (15, 21) or per cell basis (4, 16) using conversion factors previously published. General references to the micromethodology are References 7 and 13.

RESULTS

On a protein basis, the specific activity of fumarase was about 4-fold higher in guard cells than in the other cell types assayed (Fig. 1). On a Chl basis, fumarase-specific activity was 3- to 7-fold higher in guard cells than in the mesophyll (Table II). However, on a cell basis, fumarase-specific activity was lower in guard cells than in the mesophyll.

The specific activity (protein or Chl basis) of 6-P-gluconate dehydrogenase was higher in guard cells than in the epidermis or mesophyll (Fig. 1, Table I). On a weight basis, the specific activities were similar in all cell types (155 to 240 mmol/kg [dry weight]·h, Table II).

(NAD) glyceraldehyde-P dehydrogenase-specific activities were the highest of the enzymes assayed (Fig. 1, Table II). On a protein or Chl basis, the specific activity was highest in guard cells, although, on an absolute basis, there was more activity in mesophyll cells.

[1] Supported by a Department of Energy grant to W. H. O., Jr. and a travel grant from the Deutsche Forschungsgemeinschaft to R. H.

[2] Permanent address: Technische Universität München, Lehrstuhl für Botanik, Arcisstraße 21, D-8000 München 2, F. R. G.

Table I. *Analytical Flow Chart for Initial Steps of Enzyme Assays*

Enzyme	Specific Step			First Indicator Step	
	Reagent	Blank/standard	Termination	Reagent	Termination
	mM			*mM*	
Fumarase (EC 4.2.1.2)	0.2 μl 50 imidazole-Cl (pH 7), 10 fumarate; 0.02% (w/v) Triton X-100 for 60 min at 25°C	−Fumarate/ malate	pH shift by next reagent	0.2 μl 250 2-amino-2-methylpropanol (pH 10.1), 0.4 NAD^+, 20 glutamate; 2.5 μg/ml malic dehydrogenase, 2.0 μg/ml glutamate-oxaloacetate transaminase for 30 min at 25°C	1 μl 0.12 N NaOH followed by 80°C for 20 min
6-P-gluconate dehydrogenase (EC 1.1.1.44)	0.2 μl 50 Tes (pH 7.5), 10 $MgCl_2$, 0.2 $NADP^+$, 1 6-P-gluconate for 45 min at 25°C	−6-P-gluconate/ NADPH	1 μl 0.12 N NaOH followed by 80°C for 20 min	None	
(NAD) glyceraldehyde-P dehydrogenase (EC 1.2.1.12)	0.2 μl 100 Tris-Cl (pH 7.8), 10 $MgCl_2$, 10 2-mercaptoethanol, 0.5 ATP, 0.2 NADH, 2 3-P-glycerate; 0.5 μg/ml dialyzed P-glycerate kinase for 60 min at 25°C	−3-P-glycerate/ NAD^+	1 μl 0.12 N HCl followed by 80°C for 20 min	None	
Hydroxypyruvate (glyoxylate) reductase (EC 1.1.1.81)	0.2 μl 100 Mes (pH 6), 0.4 NADH, 250 glyoxylate; 0.01% (w/v) Triton X-100 for 60 min at 25°C	−Glyoxylate/ NAD^+	1 μl 0.15 N HCl followed by 80°C for 20 min	None	
Ribulose-bisP carboxylase (EC 4.1.1.39)	0.1 μl (or 0.5 μl) 50 Tris-Cl (pH 8.1), 10 $MgCl_2$, 1 GSH, 20 $NaHCO_3$, 0.2 ribulose-bisP; 0.02% (w/v) BSA for 15 min at 25°C	−Ribulose-bisP/ 3-P-glycerate	pH shift by next reagent	0.1 μl (or 0.5 μl) 100 imidazole Cl (pH 6.6), 0.03 NADH, 0.1 EDTA, 0.3 ATP, 20 NaCl; 100 μg/ml dialyzed (NAD) glyceraldehyde-P dehydrogenase, 40 μg/ml dialyzed 3-P-glycerate kinase for 15 min at 25°C	1 μl 0.3 N HCl followed by 80°C for 20 min

Neither ribulose-bisP carboxylase nor hydroxypyruvate reductase was detected in guard cells or epidermis (Fig. 1, Table II). There was no striking difference in the distribution of the specific activities of these two enzymes between the mesophyll cell types.

DISCUSSION

It was reported earlier that enzymes of the photosynthetic carbon reduction pathway are present in both mesophyll cell types (15, 19). These enzyme activities are insignificant in guard cells (8, 19, 23; W. H. Outlaw, Jr., M. C. Tarczynski, L. Anderson, unpublished) and ordinary epidermal cells (19) of C_3 plants. (There is immunological evidence, however, for ribulose-bisP carboxylase in guard cells of some Crassulacean acid metabolism plants [8]). Our results (Fig. 1, Table II) confirm these previous reports and are included here for purposes of comparison. Our inability to detect hydroxypyruvate reductase, a marker enzyme for the photosynthetic carbon oxidation pathway, in cells lacking the photosynthetic carbon reduction pathway supports the conventional view (6, 26) of the relationship between these two carbon pathways. Thus, it seems clear that both these pathways are absent in guard cells of C_3 plants. However, PSII is present (11, 21, 28, but see 24).[3] Because guard cells possess PSII and lack (NADP) glyceraldehyde-P dehydrogenase, the electron flow in guard cell chloroplasts may serve to sense PAR (21). There are data that show guard cells are sensitive to PAR (25, A. Schwartz and E. Zeiger, unpublished) and that red light and blue light act synergistically in stimulating malate production in epidermal peels (12). In addition, several enzyme activities extracted from epidermal peels are modulated by thiol reagents (L. M. Rao and L. Anderson, unpublished), and thiol-inhibiting reagents prevent stomatal opening (10). Conceivably, this modulation might be effected *in situ* by linear electron transport, but we are aware that these effects may be brought about by other mechanisms.

(NAD) glyceraldehyde-P dehydrogenase activity, 6-P-gluconate dehydrogenase activity, and fumarase activity were higher in guard cells than in the other leaf cells (protein or Chl basis). If the elevated activities of these enzymes are correlated with high potential fluxes through glycolysis, the oxidative pentose-P pathway, and the tricarboxylic acid cycle, these findings indicate that

[3] Any lingering doubt that PSII is present in guard cells can be dismissed on the basis of our unpublished results (K. C. Vaughn and W. H. Outlaw, Jr.). We have demonstrated with electron microscope-level histochemistry (see 27) the presence of PSII in barley guard cells. We have also detected DCMU-sensitive variable fluorescence in individual guard cell pairs of broad bean using an ultramicorfluorometer.

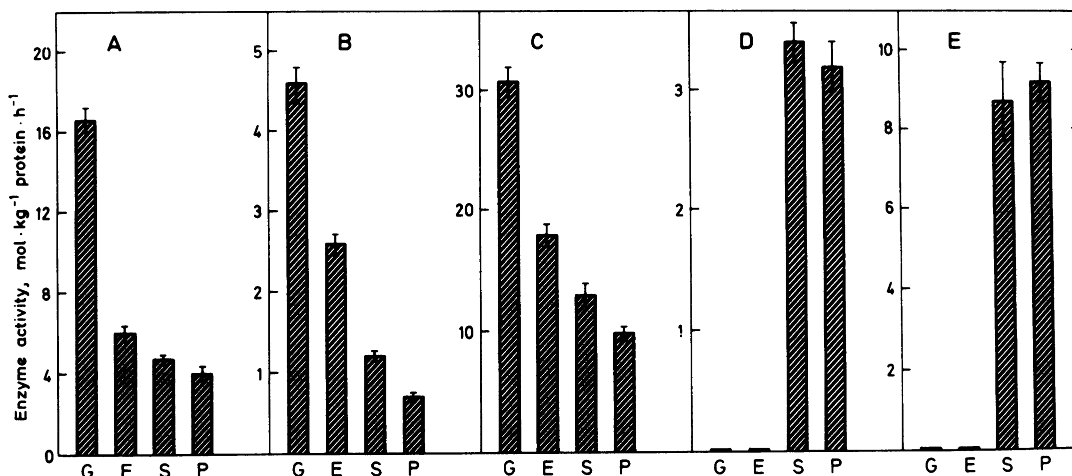

FIG. 1. Protein specific activity of fumarase (A), 6-P-gluconate dehydrogenase (B), (NAD) glyceraldehyde-P dehydrogenase (C), hydroxypyruvate reductase (D), and ribulose-bisP carboxylase (E), in guard cells (G), epidermal cells (E), spongy parenchyma cells (S), and palisade parenchyma cells (P) dissected from freeze-dried *V. faba* leaflet. Bars are SE. $n \simeq 20$ (A–D) or $n = 6$ (E).

Table II. *Enzyme Specific Activities in Guard Cells and Other Cells of V. faba L. Leaflet*

Statistics are in Figure 1.

	Fumarase	6-P-Gluconate Dehydrogenase	(NAD) Glyceraldehyde-P Dehydrogenase	Hydroxypyruvate Reductase	Ribulose-bisP Carboxylase
Guard cells					
mmol/kg (dry wt)·h	860	240	1600	N.D.[a]	N.D.
μmol/mg Chl·h	576	160	1072	N.D.	N.D.
pmol/cell pair·h	5.2	1.4	9.6	N.D.	N.D.
Epidermal cells					
mmol/kg (dry wt)·h	440	190	1300	N.D.	N.D.
Spongy parenchyma cells					
mmol/kg (dry wt)·h	820	205	2300	610	1530
μmol/mg Chl·h	186	46	522	138	347
pmol/cell·h	11	2.8	32	8.4	21
Palisade parenchyma cells					
mmol/kg (dry wt)·h	850	155	2100	670	1940
μmol/mg Chl·h	80	15	199	64	184
pmol/cell·h	9.9	1	24	9	22

[a] N.D., not detectable.

guard cells have evolved high capacity for heterotrophic energy-converting processes. This interpretation is consistent with the energy requirements for ion accumulation (*e.g.* 3), oxaloacetate reduction (*e.g.* 18), and starch catabolism (*e.g.* 17) during stomatal opening.

Acknowledgments—Drs. K. Raschke, H. Schnabl, and A. Thistle are thanked for manuscript suggestions.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. HAMPP R, H ZIEGLER 1980 On the use of *Avena* protoplasts to study chloroplast development. Planta 147: 485–494
3. HUMBLE GD, K RASCHKE 1971 Stomatal opening quantitatively related to potassium transport. Evidence from electron probe analysis. Plant Physiol 48: 447–453
4. JONES MGK, WH OUTLAW JR, OH LOWRY 1977 Enzymic assay of 10^{-7} to 10^{-14} moles of sucrose in plant tissues. Plant Physiol 60: 379–383
5. KATO T, SJ BERGER, JA CARTER, OH LOWRY 1973 An enzymatic cycling method for nicotinamide-adenine dinucleotide with malic and alcohol dehydrogenases. Anal Biochem 53: 86–97
6. LORIMER GH 1981 The carboxylation and oxygenation of ribulose-1,5-bisphosphate: The primary events in photosynthesis and photorespiration. Annu Rev Plant Physiol 32: 349–383
7. LOWRY OH, JV PASSONNEAU 1972 A Flexible System of Enzymatic Analysis Academic Press, New York
8. MADHAVAN S, BN SMITH 1982 Localization of ribulose bisphosphate carboxylase in the guard cells by an indirect immunofluorescence technique. Plant Physiol 69: 273–277
9. MATSCHINSKY FM, JV PASSONEAU, OH LOWRY 1968 Quantitative histochemical analysis of glycolytic intermediates and cofactors with an oil well technique. J Histochem Cytochem 16: 29–39
10. MOURAVIEFF I 1971 Les inhibiteurs des groupes thiols empêchent l'ouverture des ostioles somatiques. Importance probable des glycéraldéhyde phosphate déshydrogénases. Physiol Veg 9: 109–118
11. OGAWA T, D GRANTZ, J BOYER, GOVINDJEE 1982 Effects of cations and abscisic acid on chlorophyll *a* fluorescence in guard cells of *Vicia faba*. Plant Physiol 69: 1140–1144
12. OGAWA T, H ISHIKAWA, K SHIMADA, K SHIBATA 1978 Synergistic action of red and blue light and action spectra for malate formation in guard cells of *Vicia faba* L. Planta 142: 61–65
13. OUTLAW WH JR 1980 A descriptive evaluation of quantitative histochemical methods based on pyridine nucleotides. Annu Rev Plant Physiol 31: 299–311
14. OUTLAW WH JR 1982 Carbon metabolism in guard cells. Rec Adv Phytochem 16: 185–222
15. OUTLAW WH JR, CL SCHMUCK, NE TOLBERT 1976 Photosynthetic carbon metabolism in the palisade parenchyma and spongy parenchyma of *Vicia faba* L. Plant Physiol 58: 186–189
16. OUTLAW WH JR, OH LOWRY 1977 Organic acid and potassium accumulation in guard cells during stomatal opening. Proc Natl Acad Sci USA 74: 4434–4438
17. OUTLAW WH JR, J MANCHESTER 1979 Guard cell starch concentration quantitatively related to stomatal aperture. Plant Physiol 64: 79–82
18. OUTLAW WH JR, J MANCHESTER, CA DICAMELLI 1979 Histochemical approach to properties of *Vicia faba* guard cell phosphoenolpyruvate carboxylase. Plant Physiol 64: 269–272
19. OUTLAW WH JR, J MANCHESTER, CA DICAMELLI, DD RANDALL, B RAPP, GM VEITH 1979 Photosynthetic carbon reduction pathway is absent in chloroplasts of *Vicia faba* guard cells. Proc Natl Acad Sci USA 76: 6371–6375

20. OUTLAW WH JR, J MANCHESTER, VE ZENGER 1981 The relationship between protein content and dry weight of guard cells and other single cell samples of *Vicia faba* L. leaflet. Histochem J 13: 329–336
21. OUTLAW WH JR, BC MAYNE, VE ZENGER, J MANCHESTER 1981 Presence of both photosystems in guard cells of *Vicia faba* L. Implications for environmental signal processing. Plant Physiol 67: 12–16
22. PEARSON CJ, FL MILTHORPE 1974 Structure, carbon dioxide fixation and metabolism of stomata. Aust J Plant Physiol 1: 221–236
23. SCHNABL H 1981 The compartmentation of carboxylating and decarboxylating enzymes in guard cell protoplasts. Planta 152: 307–313
24. SCHNABL H, R HAMPP 1980 *Vicia* guard cell protoplasts lack photosystem II activity. Naturwissenschaften 67: 465–466
25. SHARKEY TD, K RASCHKE 1979 Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. Plant Physiol 68: 1170–1174
26. TOLBERT NE 1981 Metabolic pathways in peroxisomes and glyoxysomes. Annu Rev Biochem 50: 133–157
27. VAUGHN KC, SO DUKE 1981 Cytochemical localization of photosystem II donor sites. Histochemistry 73: 363–369
28. ZEIGER E, P ARMOND, A MELIS 1980 Fluorescent properties of guard cell chloroplasts. Plant Physiol 67: 17–20