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Calvin-Benson Cycle Enzymes in Guard-Cell Protoplasts from *Vicia faba* L.

Implications for the Greater Utilization of Phosphoglycerate/Dihydroxyacetone Phosphate Shuttle between Chloroplasts and the Cytosol

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ABSTRACT

Activities of Calvin-Benson cycle enzymes were found in protoplasts of guard cells from *Vicia faba* L. The activities of NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPD) and ribulose-1,5-bisphosphate carboxylase (RuBPC) were 2670 and 52 micromoles per milligrams chlorophyll per hour, respectively. Activities of NADP-GAPD and RuBPC in guard cells were increased by red light illumination, and the light activations were inhibited completely by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II. Enzymes related to the Calvin-Benson cycle such as 3-phosphoglycerate kinase (PGAK), triose phosphate (TP) isomerase, and fructose-1,6-bisphosphatase (FBPase) were shown to be present in guard-cell chloroplasts. From these results, we conclude that the photosynthetic carbon reduction pathway is present in guard-cell chloroplasts of *Vicia faba*. We compared these enzyme activities in guard cells with those in mesophyll cells. The activities of NADP-GAPD and PGAK were more than several-fold higher and that of TP isomerase was much higher in guard-cell chloroplasts than in mesophyll chloroplasts. In contrast, activities of RuBPC and FBPase were estimated to be roughly half of those in mesophyll chloroplasts. High activities of PGAK, NAD-GAPD, and TP isomerase were found in fractions enriched in cytosol of guard cells. Illumination of guard-cell protoplasts with red light increased the cellular ATP/ADP ratio from 5 to 14. These results support the interpretation that guard cells utilize a shuttle system (*e.g.* phosphoglycerate [PGA]/dihydroxyacetone phosphate [DHAP] shuttle) for an indirect transfer of ATP and reducing equivalents from chloroplasts to the cytosol.

Stomata open in response to red light. This red light-induced stomatal opening is mediated at least partly by chloroplasts in guard cells and is related to K^+ accumulation in guard cells (9). The chloroplasts may provide energy for such ion uptake through cyclic and noncyclic photophosphorylations (31). However, the role of guard-cell chloroplasts in stomatal opening is still obscure. Stomata open when CO_2 is removed from air (22, 37). It is, therefore, of great importance to know whether guard-cell chloroplasts can fix CO_2 photosynthetically, since the presence of photosynthetic CO_2 fixation decreases the CO_2 concentration surrounding guard cells.

There are conflicting reports about the capacity of photosynthetic carbon reduction in guard-cell chloroplasts (17, 38). Several lines of recent evidence indicate that the Calvin-Benson cycle does not operate in guard-cell chloroplasts. Willmer and Dittrich (35) found that the first products of $^{14}CO_2$ fixation in the light were malate and aspartate in epidermis of *Tulipa* and *Commelina*. Raschke and Dittrich (23) confirmed this and found neither radioactive 3-PGA[1], a primary product of photosynthetic CO_2 fixation, when epidermal tissue of the same plant species was exposed to $^{14}CO_2$, nor RuBPC activity. Schnabl (25) did not find any labeled phosphorylated product of the Calvin-Benson cycle when *Vicia* guard-cell protoplasts were exposed to $^{14}CO_2$ in the light. Moreover, Outlaw *et al.* (18) reported that the guard cell lacked activities of RuBPC and Ru5PK, Calvin-Benson cycle enzymes, and they also demonstrated the absence of RuBPC protein by immunoelectrophoresis. Although low levels of RuBPC in guard cells were subsequently found (7, 26), these were suggested to be insignificant or due to contamination by mesophyll cells (17).

Recently, Zemel and Gepstein (40) reported the presence of RuBPC in *Vicia* guard-cell protoplasts using immunofluorescence and immunoblotting. They suggested that the failure to detect RuBPC in the earlier studies was due to high proteolytic activity in guard cells. Shimazaki and Zeiger (32) found that red, light-dependent CO_2 uptake and concomitant O_2 evolution, signs of photosynthetic CO_2 fixation, were inhibited by DCMU in *Vicia* guard-cell protoplasts. Most recently, Gotow *et al.* (4) found that PGA, sugar monophosphate, sugar diphosphate, and triose phosphate were labeled with ^{14}C in guard-cell protoplasts when they were exposed to $^{14}CO_2$ under red light. All these data reveal that the Calvin-Benson cycle does operate in guard-cell chloroplasts under

[1] Abbreviations: PGA (PGAK), 3-phosphoglycerate (kinase); DHAP, dihydroxyacetone phosphate; FBPase, fructose-1,6-bisphosphatase; GAP, glyceraldehyde-3-phosphate; NAD(P)-GAPD, NAD(P)-glyceraldehyde-3-phosphate dehydrogenase; NAD(P)-MDH, NAD(P)-malate dehydrogenase; PEP (PEPC), phosphoenolpyruvate (carboxylase); RuBP (RuBPC), ribulose-1,5-bisphosphate (carboxylase); Ru5P (Ru5PK), ribulose-5-phosphate (kinase); TP, triose phosphate.

red light illumination. However, there is no evidence for the enzyme activities of the Calvin-Benson cycle in guard-cell chloroplasts except for recent reports on NADP-GAPD in *Vicia* (7, 20), and NADP-GAPD, FBPase, and TP isomerase in *Commelina* (24).

In the present study, we have provided evidence for the enzyme activities of RuBPC, which is unique to the Calvin-Benson cycle, and PGAK, NADP-GAPD, TP isomerase, and FBPase in chloroplasts of *Vicia* guard cells. We have also been able to show that red light activates NADP-GAPD and RuBPC. On the basis of the enzyme activities found in this study, we suggest a role of guard-cell chloroplasts in stomatal opening.

MATERIALS AND METHODS

Plant Material

Plants of *Vicia faba* L. (cv Otafuku) were grown hydroponically under natural light, as described previously (30).

Preparation of Protoplasts

Guard-cell protoplasts were isolated enzymically from the lower epidermis of 4- to 5-week-old leaves of *Vicia*, as described previously (28, 30). The isolated protoplasts were suspended in 0.4 M mannitol and 1 mM $CaCl_2$ and kept on ice until use under dim light. The purity of guard-cell protoplast preparations was examined microscopically. No mesophyll protoplasts were found in bright-field micrographs of representative guard-cell protoplast preparations (2000 cells). Inspection of the same protoplast samples by fluorescence microscopy showed no Chl fluorescence other than guard-cell protoplasts, indicating that there was no detectable contamination by mesophyll protoplasts or mesophyll fragments. Mesophyll protoplasts were isolated from the *Vicia* leaves according to the previous method (28). Fully expanded leaf segments devoid of the lower epidermis were digested in the maceration medium containing 0.5% Macerozyme R-10 (Yakult Pharmaceutical Industry Co.), 2% Cellulase Onozuka R-10, 0.5% sodium dextran sulfate, 0.2% bovine serum albumin, 1 mM $CaCl_2$, and 0.6 M mannitol at pH 5.5. The isolated protoplasts were stored in 0.65 M mannitol and 1 mM $CaCl_2$ on ice.

Assay for Enzyme Activities

All enzyme activities were measured at 23°C by oxidation or reduction of NADH (or NADPH) at 340 nm with a Hitachi 557 dual-wavelength spectrophotometer. All activities were estimated by the subtraction of control values (without substrate) and were expressed in terms of the catalytic rates for specific substrates (*e.g.* PGA, RuBP) on a Chl basis. Some reactions were initiated by adding the specific substrates to the assay mixtures (total, 1 mL), which contained guard-cell protoplasts or mesophyll protoplasts, or the crude protoplast extracts. The protoplasts and crude extracts were included in the mixture just before the addition of substrates. Other reactions were started by adding 50-μL aliquots of the protoplast suspension to the assay mixtures. The measurements were done for 10 min for RuBPC and FBPase and within 5 min for other enzymes.

NADP-GAPD was assayed according to the method of Wolosiuk and Buchanan (36). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM PGA, 1 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADPH, and 1 IU PGAK. NAD-GAPD was measured in a similar manner by replacing NADPH with NADH. RuBPC was assayed by the method of Racker (21), slightly modified from the medium used by Lorimer *et al.* (14). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 1 mM RuBP, 1 mM ATP, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 1 IU PGAK, 1 IU NAD-GAPD, 1 IU TP isomerase, and 2 IU α-glycerophosphate dehydrogenase. FBPase was measured according to Kelly *et al.* (10). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 1 mM FBP, 1 mM EDTA, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 4 IU of glucosephosphate isomerase, and 2 IU of glucose-6-phosphate dehydrogenase. PGAK was assayed by coupling with NAD-GAPD (2). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM PGA, 2 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADH, and 1 IU NAD-GAPD. TP isomerase was assayed according to Latzko and Gibbs (13). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 0.2 mM NADH, 2 mM GAP, and 2 IU α-glycerophosphate dehydrogenase. The reaction was started by the addition of GAP. PEPC was measured according to Hatch and Oliver (6) with slight modification. The contribution of PEP carboxykinase activity was not corrected because such activity seemed to be undetectable (3, 28). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 5 mM $KHCO_3$, 2.5 mM PEP, 0.2 mM NADH, and 2 IU NAD-malate dehydrogenase (NAD-MDH). The assay mixture for NADP-MDH consisted of 100 mM Tris-HCl (pH 7.8), 0.2 mM NADPH, 2 mM oxaloacetic acid (OAA), and 2 mM EDTA. In all reactions, 0.05% Triton X-100 was present unless otherwise stated.

Activation of Enzymes by Red Light

Enzymes of the Calvin-Benson cycle were activated at 23°C by illumination of protoplast suspensions with red light at 800 μmol m^{-2} s^{-1}, which was obtained by passing the light through a red cut-off glass filter (Corning 2-61), a Cinemoid 5A film, and a 50-mm water layer. The light source was a halogen lamp (Sylvania EXR, 300 W). Guard-cell protoplasts were suspended in 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 10 mM Mes-NaOH (pH 6.2, 0.8 mL). Mesophyll protoplasts were suspended in 0.65 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 10 mM Mes-NaOH (pH 6.2, 0.8 mL). Enzyme activities were measured immediately after the illumination by transferring 50-μL aliquots of the protoplast suspension from the incubation medium to the reaction mixtures. Fluence rate was measured with a radiometer (model 188B LI-COR Inc.). DCMU was added at 10 μM.

Fractionation of Guard-Cell Protoplasts

Guard-cell protoplasts were fractionated into chloroplastic and extrachloroplastic materials by forcing the protoplasts through a narrow-aperture (5 μm) nylon mesh according to Shimazaki and Zeiger (31) with slight modifications. Guard-

cell protoplasts in 0.4 M mannitol and 1 mM $CaCl_2$ were mixed with the same volume of 0.2 M mannitol, 20 mM Tricine-NaOH (pH 7.5), and 2 mM EDTA in order to cause them to swell. After keeping this suspension for 2 min, the protoplasts (0.5 mL) were poured onto a 5-μm nylon mesh, which was fixed at the top of a microfuge tube (1.5 mL), and ruptured by rapid centrifugation (12,000 rpm, 60 s) in a microfuge (Tomy Seiko Co., Ltd., MR-15A). The resulting pellet was gently homogenized with soft silicone rubber to disrupt the protoplasts that passed through the nylon mesh, then separated again into chloroplastic and extrachloroplastic fractions by centrifugation (3,000 rpm, 10 min). This procedure reduced the amount of cytosolic contamination in chloroplasts. The cytosolic supernatant was removed with a Pasteur pipette. The chloroplast pellet was suspended in 0.3 M mannitol, 10 mM Tricine-NaOH (pH 7.5), 1 mM EDTA, and 0.5 mM $CaCl_2$ and was homogenized uniformly in a Teflon homogenizer. All preparations were done at 0°C.

Adenylate Level

ATP was determined by the luciferin-luciferase method, as described previously (29). ADP was estimated as ATP by enzymic conversion to ATP. The conversion mixture (0.4 mL) contained the adenylate solution, 20 mM $MgSO_4$, 1 mM PEP, and 50 μg pyruvate kinase. The reaction was conducted at 37°C for 1 h. Guard-cell protoplasts (0.2 mL) suspended in 0.4 M mannitol, 0.5 mM Mes-NaOH (pH 6.2), 10 mM KCl, and 1 mM $CaCl_2$ were illuminated with red light (700 μmol m^{-2} s^{-1}) for 1 min in a similar manner described above. The reaction was stopped by the addition of 0.2 M ice-cold $HClO_4$ (0.7 mL) to protoplast suspension, and then the mixture was allowed to stand at 0°C for 5 min for complete adenylate extraction. The extracted adenylate solution was neutralized by adding an appropriate volume of 0.5 M KOH, and the pH of the solution was adjusted by the addition of 0.2 M phosphate buffer (pH 7.4, 0.5 mL). After centrifugation (3000 rpm, 10 min), the supernatant was used for ATP determination and an aliquot of the supernatant was used for the determination of ADP.

Chl Concentration

Chl was determined by the method of Arnon (1). Since the Chl concentration in the cytosolic fraction was very low, it was estimated by the intensity of Chl *a* fluorescence in the presence of DCMU (10 μM). Fluorescence was measured according to the previous method at 60 μmol m^{-2} s^{-1} of actinic light (28).

Chemicals

NAD-GAPD, yeast PGAK, rabbit muscle pyruvate kinase (type III), α-glycerophosphate dehydrogenase (type X) and TP isomerase (type III-S) were purchased from Sigma. NAD-MDH (type II) was obtained from Toyobo (type II).

RESULTS

Enzyme Activities

Figure 1 shows the time course of the NADP-GAPD reaction. An initial absorbance increase suggests the release of UV-absorbing substances from guard cells, and a subsequent absorbance decrease indicates NADPH oxidation (Fig. 1, c, d, e, f). No NADP-GAPD activity or release of UV-absorbing substances was observed when Triton X-100 was omitted (29) or when its concentration was very low (Fig. 1, a and b). The activity reached a maximum at 0.02% Triton X-100 (Fig. 1d). Triton X-100 was required for the release of enzyme upon the lysis of the protoplasts. Unless otherwise stated, we measured all enzyme activities with Triton X-100 at 0.05% in both guard-cell and mesophyll protoplasts.

NADP-GAPD activities were 2670 μmol mg^{-1} Chl h^{-1} in guard-cell protoplasts and 630 μmol mg^{-1} Chl h^{-1} in mesophyll protoplasts (Fig. 2). In guard-cell protoplasts, no activity was detected when one of the components (PGA, NADPH, or ATP) was omitted from the reaction mixture. PGAK was not required for this reaction, probably due to a high activity of endogenous PGAK in guard cells (Table II). NADP-GAPD activity was slightly reduced in most cases when DTT was omitted from the assay mixture (Fig. 2). In such guard-cell preparations (Fig. 2; Table I, Experiment 1, a), when the cells were lysed and preincubated in the reaction mixture for 5 min, NADP-GAPD activity decreased to less than 20% of the original activity (Table I, Experiment 1, b), but the activity

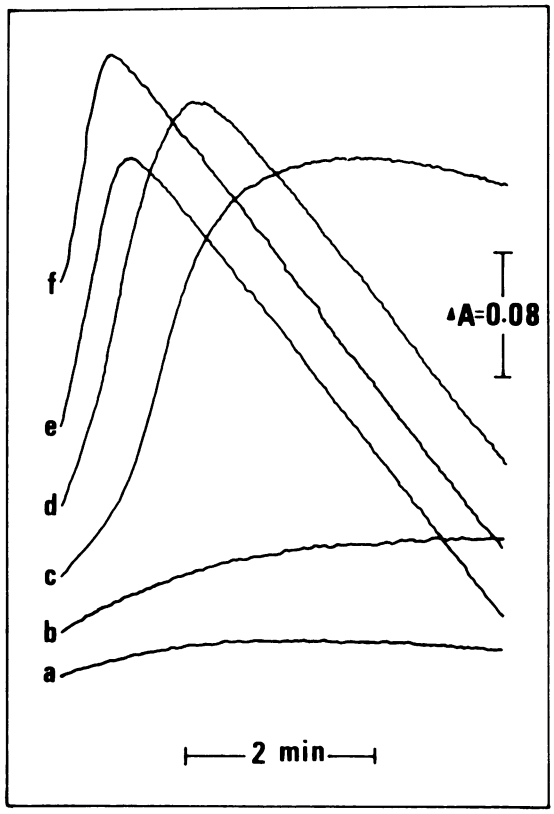

Figure 1. Triton X-100 requirement for measurement of the NADP-GAPD activity in *Vicia* guard-cell protoplasts. Triton X-100 was added to the reaction mixture (1 mL) at 0 (a), 0.005 (b), 0.01 (c), 0.02 (d), 0.05 (e), and 0.1% (f). Enzyme reaction was initiated by the addition of guard-cell protoplast suspension. The reaction mixture (1 mL) contained 0.44 μg of Chl. NADP-GAPD activity was 1690 μmol mg^{-1} Chl h^{-1} in the presence of 0.05 Triton X-100.

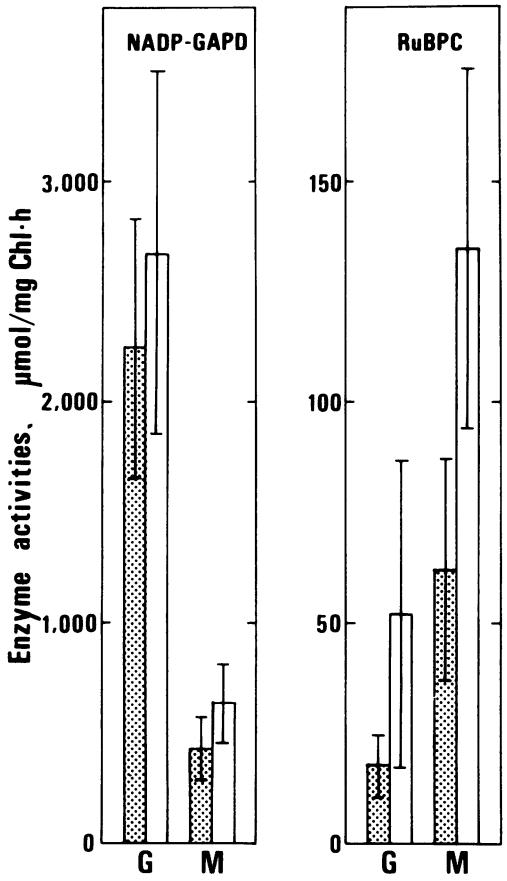

Figure 2. Enzyme activities of NADP-GAPD and RuBPC in guard-cell and mesophyll protoplasts from *Vicia*. G, guard-cell protoplast; M, mesophyll protoplast. Open bar, + DTT; screened bar, − DTT. DTT was added at 4 mM. Reactions were started by the addition of PGA for NADP-GAPD and RuBP for RuBPC. The reaction mixture (1 mL) contained 0.2 to 0.6 μg Chl for guard-cell protoplasts and 3 to 6 μg Chl for mesophyll protoplasts. Values were mesans of four experiments for guard-cell RuBPC and eight experiments for mesophyll RuBPC, and six experiments for guard-cell and mesophyll NADP-GAPD. Bars represent the standard deviations.

Table I. *NADP-GAPD Activity in the Presence and Absence of DTT in Guard-cell Protoplasts from Vicia faba*

Expt.	NADP-GAPD Activity		
	−DTT	+4 mM DTT	+DTT/−DTT
	μmol mg⁻¹ Chl h⁻¹		*ratio*
1	2640[a]	3400[a]	1.29
	470[b]	3420[c]	7.28
2	1020[a]	3290[a]	3.22

[a] Guard-cell protoplasts were included in the reaction mixture just before the addition of PGA. [b] Guard-cell protoplasts were preincubated in the reaction mixture including 0.05% Triton X-100 (without PGA) for 5 min at 23°C, then the reaction was initiated by PGA. [c] After the preincubation and assay, DTT was added to the same reaction mixture and the mixture was measured again.

Table II. *Enzyme Activities of FBPase, PGAK, TP Isomerase and NAD-GAPD in Guard-Cell and Mesophyll Protoplasts from Vicia faba*

Data represent means from three to six experiments. DTT was at 4 mM. In a typical experiment, the reaction mixture (1 mL) contained 0.2–0.5 μg Chl of guard-cell protoplasts and 3–5 μg Chl of mesophyll protoplasts, respectively.

Enzyme	Guard Cells		Mesophyll Cells	
	−DTT	+DTT	−DTT	+DTT
	μmol mg⁻¹ Chl h⁻¹			
FBPase	160	170[a]	94	160[b]
PGA kinase	16,840	20,390[c]	900	1,430[d]
TP isomerase	133,300	131,100[e]	3,840	3,610[f]
NAD-GAPD		10,730[g]		500[h]

Ranges of enzyme activities: [a] 140–190, [b] 130–190, [c] 15,040–28,470, [d] 990–1,900, [e] 98,360–151,280, [f] 1,950–4,930, [g] 6,140–15,140, [h] 420–560.

was increased greatly (7-fold) by the addition of DTT (Table I, Experiment 1, c). This suggests that most of the protein molecules of NADP-GAPD existed in the active state in these protoplast samples, and they were converted to an inactive state during the preincubation.

In some preparations, NADP-GAPD activity was low when DTT was omitted (Table I, Experiment 2), suggesting that the major proportion of the NADP-GAPD molecules were in the inactive state. The conditions that determine the state of the enzyme were not clarified here.

Mean RuBPC activities were 52 μmol mg^{-1} Chl h^{-1} in guard-cell protoplasts and 130 μmol mg^{-1} Chl h^{-1} in mesophyll protoplasts (Fig. 2). RuBPC activity showed a wide range in both types of protoplasts. The highest activity of RuBPC was 93 μmol mg^{-1} Chl h^{-1} and the lowest 31 μmol mg^{-1} Chl h^{-1} in guard-cell protoplasts. Enzyme activities were reduced by the omission of DTT in both protoplast samples (Fig. 2). RuBPC activity was very low in guard cells. We incubated the enzyme with $NaHCO_3$ (10 mM) and Mg^{2+} (20 mM) for 5 and 15 min at room temperature before measurement, but there was no stimulation of the activity. Addition of PMSF, a potent protease inhibitor, failed to increase the RuBPC activity.

FBPase activities were 170 μmol mg^{-1} Chl h^{-1} in guard-cell protoplasts and 160 μmol mg^{-1}Chl h^{-1} in mesophyll protoplasts (Table II). Activities were reduced by the omission of DTT in mesophyll cells, but not in guard cells.

Extremely high activities of PGAK and TP isomerase, components of the Calvin-Benson cycle but not unique to it, were found in guard-cell protoplasts (Table II). High NAD-GAPD activity was also found (Table II). High NAD-GAPD activity has previously been reported in guard cells of *Vicia* (5) and *Commelina* (24).

Activation of the Enzymes by Red Light

When guard-cell protoplasts were illuminated with red light, NADP-GAPD activity increased, with the activation exhibiting large variations (130–240%) from sample to sample. Figure 3a illustrates the most prominent example. The activity reached a maximum within 15 min, and it declined to the

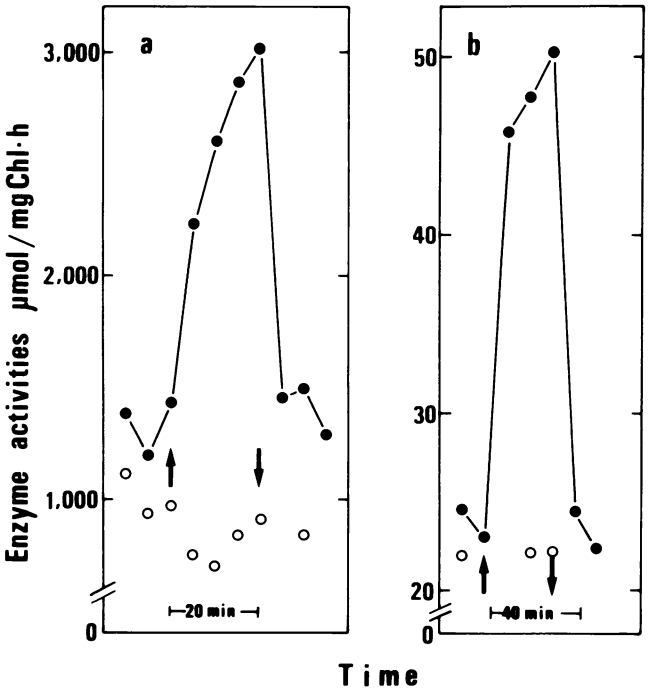

Figure 3. Activation of NADP-GAPD and RuBPC by red light in *Vicia* guard-cell protoplasts. a, NADP-GAPD; b, RuBPC. (●), −DCMU; (○), +DCMU. DCMU was added at 10 μM. Guard-cell protoplast suspensions (0.8 mL) were illuminated with red light at 800 μmol m^{-2} s^{-1}. The reaction mixtures for the enzyme assay (1 mL) contained 0.2 to 0.5 μg of Chl.

initial level within 10 min upon turning off the red light. This light activation was inhibited completely by DCMU, an inhibitor of PSII. Essentially the same results were obtained with RuBPC of guard-cell protoplasts (Fig. 3b). RuBPC activity was increased to 200% with red light, but not in the presence of DCMU.

Localization of Enzyme Activities

Guard-cell protoplasts were fractionated into chloroplastic and extrachloroplastic materials. Seventy-five percent of NADP-MDH, a marker for chloroplast stroma (3), was recovered in the pellet. Ninety percent of Chl, a marker for thylakoid membrane, was retained in the pellet. More than 90% of PEPC activity, a marker for cytosol, was recovered in the supernatant (Table III, experiment 1). These results indicate that chloroplasts were separated from the rest of the cellular components and that chloroplasts and cytosol were enriched in the pellet and supernatant fractions, respectively. In the chloroplast fraction, recovery of NADP-MDH was lower than that of Chl. This lower recovery was probably due to breakage of some chloroplasts when passed through the nylon mesh and release of stromal enzymes into the supernatant, even though the chloroplasts and their fragments were sedimented in the pellet (7). Contamination of the chloroplasts by the remaining cytoplasmic components was estimated to be 8.5%; contamination of the cytosol by the chloroplastic enzymes was about 25% (Table III, experiment 1).

Most NADP-GAPD activity was recovered in the pellet,

Table III. *Distribution of PEPC, NADP-MDH, Chl, NADP-GAPD, NAD-GAPD, PGAK, TP Isomerase and FBPase in Chloroplast- and Cytosol-Enriched Fractions of Vicia Guard-Cell Protoplasts.*

Enzyme or Marker	Relative Activity	
	Chloroplast	Supernatant
	%	
Experiment 1[a]		
PEPC	8.5	91.5
NADP-MDH	74.6	25.4
Chl	90.2	9.8
NADP-GAPD	61.6	38.4
PGAK	30.1	69.9
TP isomerase	36.4	63.6
NAD-GAPD	14.6	85.4
Experiment 2[b]		
PEPC	15.7	84.3
NADP-MDH	86.8	13.2
FBPase	49.5	50.5
NADP-GAPD	71.8	28.2

[a] Values are the averages of three experiments. [b] Values are the averages of two experiments. DTT was at 4 mM.

indicating that the major part of this enzyme was localized in the chloroplasts. The majority of the PGAK was recovered in the supernatant, but a significant amount (30%) was retained in the pellet. A major proportion of TP isomerase was found in the supernatant, with 36% of the activity retained in the pellet. On the basis of the cross-contamination, about 33% of PGAK (= 6,730 μmol mg^{-1} Chl h^{-1}) and 42% of TP isomerase (= 55,100 μmol mg^{-1} Chl h^{-1}) were estimated to be localized in chloroplasts (Table II; Table III, experiment 1). Activities of PGAK and TP isomerase in guard-cell chloroplasts were estimated to be at least more than 4- and 15-fold higher than those in mesophyll chloroplasts, because measured activities in mesophyll cells consisted of both enzymes of chloroplast and cytosol (Table II). About 85% of NAD-GAPD activity was recovered in the supernatant, indicating that most of the enzyme was localized in the cytosol (Table III, experiment 1).

The distribution of FBPase activity between chloroplasts and cytosol was determined by the same method (Table III, experiment 2). Sixteen percent of PEPC, a marker for cytosol, was found in the pellet and 13% of NADP-MDH, a marker for stromal enzyme, was found in the supernatant. Fifty percent of FBPase was recovered in the pellet. These results indicate that 48% of total FBPase activity was located in the chloroplasts. Similar results have been obtained for *Commelina* guard cells by Robinson and Preiss (24). FBPase activity in *Vicia* guard-cell chloroplasts was estimated to be at least 50% of that in the mesophyll chloroplasts, because activities measured in mesophyll cells were the sum of chloroplastic and cytosolic enzymes (Table II).

A major proportion of NADP-GAPD activity was recovered again in the chloroplastic fraction in this experiment (Table III, experiment 2). NADP-GAPD activity was estimated to be more than several-fold higher in guard-cell chloroplasts than in mesophyll chloroplasts (Fig. 2).

ATP/ADP Ratio

The ratio of ATP/ADP was around 5, when guard-cell protoplasts were incubated in the darkness. Upon illumina-

tion of the protoplasts with red light, the ATP/ADP ratio increased up to 14 in 1 min (Table IV).

DISCUSSION

The present study indicates the presence of Calvin-Benson cycle enzymes in guard-cell chloroplasts of *Vicia faba*. NADP-GAPD, PGAK, and TP isomerase were present with high activities, but RuBPC and FBPase activities were low. We measured the Calvin-Benson cycle enzymes in *Vicia* mesophyll protoplasts using the same methods. NADP-GAPD, PGAK, TP isomerase, and FBPase showed the activities similar to those reported in spinach mesophyll chloroplasts by Latzko and Gibbs (12), except for higher RuBPC activity. The high RuBPC activity is probably due to the improvement of the assay technique for RuBPC developed by Lorimer *et al.* (14). These observations verify the enzyme activities of the Calvin-Benson cycle in guard-cell chloroplasts.

Our results indicate that the activity of RuBPC in guard-cell chloroplasts is 40% of that in mesophyll chloroplasts on a Chl basis (Fig. 2). Zemel and Gepstein (40) demonstrated that RuBPC protein in guard cells was 40 to 50% of the value for mesophyll RuBPC on the same basis. Vaughn (33) found that RuBPC concentration in guard cells was 0.48% of that in mesophyll cells of *Pelargonium* on a cell basis. Assuming that *Pelargonium* guard cells contain much less Chl than mesophyll cells, as has been shown in *Vicia* guard cells (1.27% of mesophyll cells) (29), the content of RuBPC in guard cells corresponds to 38% (0.48/1.27) of that in mesophyll cells on a Chl basis. These immunochemical estimations agree very well with the enzyme activity of guard-cell RuBPC.

Shimazaki and Zeiger (32) showed that O_2 was evolved in parallel with red light-dependent medium alkalinization in guard-cell protoplasts of *Vicia*, and calculated a photosynthetic CO_2 fixation up to 190 μmol CO_2 mg^{-1} Chl h^{-1} in the steady state. The high rate of CO_2 fixation cannot be explained by the relatively low RuBPC activity in guard-cell protoplasts described above. The possibility cannot be excluded that RuBPC activity in guard cells is much higher than that presented here, because measured RuBPC activities had the widest ranges, and the activity in mesophyll cells showed lower values than usually expected (Fig. 1). Optimal conditions may be needed to obtain the maximal activity of RuBPC in guard cells.

Outlaw *et al.* (19) and other workers (5, 7, 26) did not find significant activity of RuBPC in guard cells. These results conflict markedly with the present findings. One explanation for failure to detect the enzymes is proteolysis of the enzymes in guard cells (40). RuBPC seems to be more labile in guard cells than in mesophyll cells. Our results further indicate that Triton X-100 was essential for the release of Calvin-Benson cycle enzymes from guard-cell protoplasts. In the previous study (29), no activity of NADP-GAPD was found in *Vicia* guard-cell protoplasts when Triton X-100 was omitted from the reaction mixture, suggesting caution is needed when measuring the enzymes of the Calvin-Benson cycle in guard cells.

Table IV. *Cellular ATP/ADP Ratios in Guard-Cell Protoplasts in the Dark and under Red Light Illumination*

Representative values are indicated for three independent experiments. Fluence rate of red light was 700 μmol m^{-2} s^{-1}.

Expt.	ATP/ADP Ratio	
	Darkness[a]	Red light[b]
1	5.3	14
2	4.2	9.9
3	4.5	8.1

[a] Periods of dark incubation were 15 min at 24°C. [b] Red light was illuminated for 1 min after the dark incubation.

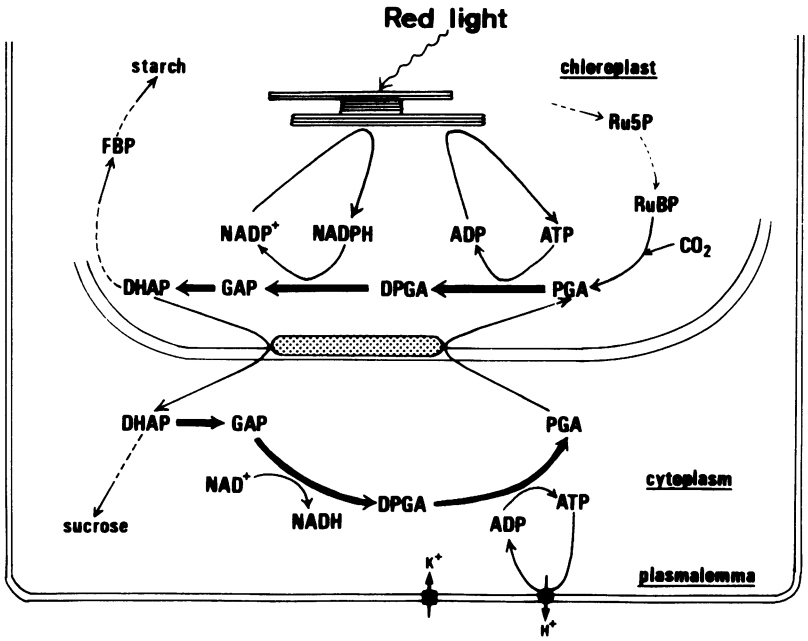

Figure 4. Proposed sequence of events in *Vicia* guard cells during stomatal opening under red light illumination. The bold lines represent enzyme reactions whose high activities were found in *Vicia* guard cells. Screened area, phosphate translocator; (●), H^+-ATPase; (■), K^+-channel; DPGA, 1,3-diphosphoglyceric acid; GAP, glyceraldehyde-3-phosphate. For details see "Discussion."

Further investigation will be needed to elucidate the discrepancy.

For the operation of the Calvin-Benson cycle, ATP, NADPH, 13 enzymes, and light activation of enzymes are required. Guard-cell chloroplasts produce ATP through cyclic and noncyclic photophosphorylations (31) and may generate NADPH on the reducing side of PSI (19, 28, 39). In this study we found high activities of PGAK, the initial ATP-consuming enzyme, and NADP-GAPD, the NADPH-consuming enzyme in guard-cell chloroplasts. We also demonstrated the presence of RuBPC, a key enzyme that traps CO_2 with RuBP. FBPase and TP isomerase were also shown to be present in guard-cell chloroplasts. Furthermore, light activations of RuBPC and NADP-GAPD through photosynthetic electron flow were observed (Fig. 3). These results indicate that the Calvin-Benson cycle operates in guard-cell chloroplasts.

The activities of Calvin-Benson cycle enzymes in guard-cell chloroplasts suggest that it will be difficult to find ^{14}C-labeled 3-PGA, when they are exposed to $^{14}CO_2$; with high activities of PGAK and NADP-GAPD on the one hand, and low activity of RuBPC on the other, ^{14}C-3-PGA would be rapidly converted to other metabolites (Fig. 4). It will be more difficult to find ^{14}C-3-PGA in guard cells under white light (which includes blue light) than under red light, because blue light-dependent metabolism proceeds preferentially under these conditions (32, 38). Experimental observations previously reported are consistent with this (23, 25, 35).

Activities of NADP-GAPD, PGAK, and TP isomerase in guard-cell chloroplasts were higher than in mesophyll chloroplasts, but RuBPC and FBPase activities were half or less than half of those in mesophyll chloroplasts. These differences in the enzyme activities between the two cell types suggest that guard cells have greatly utilized a shuttle system for indirect transfer of ATP and NADH (NADPH) between chloroplast and cytosol (8, 11), although the phosphate translocator has not yet been demonstrated in guard-cell chloroplasts (Fig. 4). If this is the case, once 3-PGA is formed in guard-cell chloroplasts by photosynthetic CO_2 fixation or by transfer from the cytosol, the compound will be phosphorylated (by PGAK), reduced (by NADP-GAPD), and finally converted to DHAP (by TP isomerase). DHAP will then be exported from chloroplasts to the cytosol, generating reducing equivalents and ATP there, and 3-PGA will again be produced by the series of reverse reactions which occur in the chloroplast (Fig. 4), or being used for the synthesis of sucrose which can be an osmoticum in guard cells (15). In the present study, high activities of enzymes required for these reverse reactions (PGAK, NAD-GAPD, TP isomerase) were demonstrated in the cytosol of guard cells (Tables II, III). Moreover, high activities of NADP-GAPD and TP isomerase combined with low activity of FBPase in the chloroplast might result in the accumulation of DHAP, accelerating export of DHAP from chloroplast to cytosol. A high activity of PGAK and a low RuBPC level in the chloroplasts might result in the rapid removal of PGA, stimulating import of PGA from the cytosol into chloroplast. These situations can facilitate the shuttle between chloroplast and cytosol and efficiently produce ATP and NADH (NADPH) in the cytosol under red light illumination (Fig. 4).

We obtained evidence that the illumination of guard-cell protoplasts with red light increased the ATP/ADP ratio from 5 to more than 10 (Table IV). Although this change in ATP/ADP ratio has been shown in the whole protoplast, it may reflect the cytosolic adenylate status, since a recent compartmentation analysis of guard-cell protoplasts revealed that 65% of ATP and ADP was accumulated in the cytosolic fraction (16).

The increase of ATP and reducing equivalents in the cytosol under red light will, in turn, facilitate the events that consume ATP and reducing equivalents in the cytosol, tonoplast, and plasmalemma. In keeping with this, Serrano *et al.* (27) have recently found that red light stimulated an electrogenic pump, which was fueled by ATP, in the plasmalemma of *Vicia* guard-cell protoplasts (30); the pump required orthophosphate in the cytoplasm for its maximum activation and was inhibited by DCMU. The requirement for orthophosphate is probably due to the fact that Pi is needed for the exchange of DHAP at the phosphate translocator on the inner membrane of the chloroplast envelope (8, 11). Coincident with the special function of guard cells, their chloroplasts seem to provide the major share of energy for ion transport across the plasmalemma.

Most recently, Gotow *et al.* (4) found radioactivity in PGA, sugar monophosphates, sugar diphosphates, and triose phosphates, when *Vicia* guard-cell protoplasts were exposed to $^{14}CO_2$ in the red light. They also found RuBP, a specific metabolite in the Calvin-Benson cycle, in the labeled sugar phosphate. These results suggested the operation of the Calvin-Benson cycle in guard-cell chloroplasts and were consistent with our findings. However, they showed that the rate of $^{14}CO_2$ fixation in guard-cell protoplasts was only 23 μmol mg^{-1} Chl h^{-1}, which corresponded to less than 10% of the photosynthetic O_2 evolution rate (4). This low ratio of CO_2 fixation/O_2 evolution contrasted with that of mesophyll cells. This finding implies that the major proportion of ATP and reducing equivalents, whose production was tightly coupled with O_2 evolution, was used for reactions other than photosynthetic CO_2 fixation in guard cells (see Fig. 4). Appreciable amounts of ATP and reducing equivalents may be consumed in the cytosol and plasmalemma. This is in good agreement with our present results.

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