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Short Communication

Changes in Sensitivity to Effectors of Maize Leaf Phosphoenolpyruvate Carboxylase during Light/Dark Transitions[1]

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ABSTRACT

Illumination of previously darkened maize (*Zea mays* L. cv Golden Cross Bantam T51) leaves had no effect on the concentration of phosphoenolpyruvate (PEP) carboxylase protein, but increased enzyme activity about 2-fold when assayed under suboptimal conditions (pH 7.0 and limiting PEP). In addition, sensitivity to effectors of PEP carboxylase activity was significantly altered; *e.g.* malate inhibition was reduced and glucose-6-phosphate activation was increased. Consequently, 10- to 20-fold differences in PEP carboxylase activity were observed during dark to light transitions when assayed in the presence of effectors. At pH 7.0 activity of purified PEP carboxylase was not proportional to enzyme concentrations. Below 0.7 microgram PEP carboxylase protein per milliliter, enzyme activity was disproportionately reduced. Including polyethylene glycol plus potassium chloride in the reaction mixture eliminated this discontinuity and substantially increased PEP carboxylase activity and reduced malate inhibition dramatically. Inclusion of polyethylene glycol in the assay mixture specifically increased the activity of PEP carboxylase extracted from dark leaves, and reduced malate inhibition of the enzyme from both light and dark leaves. Collectively, the results suggest that PEP carboxylase in maize leaves is subjected to some type of protein modification that affects both activity and effector sensitivity. We postulate that changes in quaternary structure (dissociation or altered subunit interactions) may be involved.

Modulation by light of PEPC[3] activity has been reported in several C_4 species, including *Amaranthus palmeri* (13) and *Salsola soda* (5, 6). The maximum change in activity with light/dark transitions is not large (usually 2- to 3-fold), and is most pronounced when assays are conducted under suboptimal conditions. However, apparent light modulation is not evident in a number of C_4 species, including *Zea mays* (5).

The mechanism of light modulation of PEPC has been postulated to involve disulfide reduction, because of the implicated essential role of sulfhydryl groups in enzyme activity (5, 7, 14). However, this mechanism has not been proven. Including reductants such as 2-mercaptoethanol in the extraction medium appears to stabilize both the light and dark forms of the enzyme, rather than to activate specifically the dark enzyme (5). Thus, the mechanism of light modulation of PEPC, when it is observed, remains unclear. Further, the time course of light activation of *S. soda* PEPC is sufficiently slow (60–90 min; Ref. 5) so as not to distinguish conclusively between posttranslational modification and synthesis/degradation of PEPC protein.

If light modulation of PEPC is an important regulatory mechanism in C_4 photosynthesis, it should be present in all, rather than only some, C_4 plants. Therefore, we reasoned that the failure to observe light activation in some C_4 plants (such as maize) may be due to unsuitable extraction and/or assay conditions. The objective of this study was to determine whether light modulation of maize PEPC should be observed, and if so, to determine the mechanism involved. Extracts from light and dark leaves were compared with respect to: (a) PEPC activity and PEPC protein (determined immunochemically), (b) sensitivity of PEPC activity to effectors, and (c) various factors that may modulate enzyme activity (*e.g.* reductant).

MATERIALS AND METHODS

Plant Growth. Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown in soil in a greenhouse. Plants, 3 to 4 weeks old, were used and mature leaf tissue was collected from plants that had been in the dark for 15 h or exposed to light (1000 $\mu E\ m^{-2}\ s^{-1}$) for 1 h at 25°C. Leaf tissue was immediately frozen in liquid N_2 and stored at −80°C prior to extraction.

Enzyme Extraction. Frozen leaf tissue (about 0.4 g fresh weight) was pulverized with liquid N_2 and then ground in a chilled mortar with sea sand, Polyclar AT (10% of leaf weight), and 2 ml of degassed extraction buffer (0.1 M Tris-HCl [pH 7.0], 1 mM EDTA, 10 mM $MgCl_2$, 20% (w/v) sorbitol, and 10 mM 2-mercaptoethanol). The extract was immediately centrifuged (10,000*g* for 30 s) and the supernatant obtained was desalted by centrifugal filtration with Sephadex G-25 equilibrated with degassed extraction medium minus 2-mercaptoethanol. The

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[3] Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; G6P, glucose-6-phosphate; I_{50}, concentration required for 50% inhibition; A_{50}, concentration required for half-maximal activation.

desalted crude extracts were assayed immediately. Deviations from this standard procedure are specified in the text. The standard reaction mixture (1 ml) contained 0.1 M Tris-HCl (pH 7.0 or 8.0), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 5 units malate dehydrogenase, and desalted enzyme extract (50–100 μl). As indicated in the text, some reaction mixtures were supplemented with 10% (w/v) PEG-6000 (Sigma) plus 0.15 M KCl. Reactions (25°C) were initiated by addition of PEP (0.5 or 2.5 mM), and A_{340} was monitored. As indicated in the text, malate or G6P were added at a final concentration of 5 mM.

Single Radial Immunodiffusion. Specific antibody against maize leaf PEPC was prepared as previously described (15), and used to quantitate PEPC protein in crude leaf extracts. Single radial immunodiffusion was performed as described by Sugiyama *et al.* (15). Purified PEPC (16) was used as the calibration standard.

RESULTS

PEPC Activity in Light and Dark Leaves. In preliminary experiments, maize leaf tissue was harvested in the light or dark and extracted in buffers containing 2-mercaptoethanol, followed by desalting into degassed buffer devoid of reductant to reduce the possibility of disulfide reduction during enzyme preparation. The extracts obtained from light and dark leaf tissue were assayed under different conditions of pH and PEP concentration and in the presence of G6P, an activator (2), and malate, an inhibitor (4, 10). Results from a typical experiment are shown in Table I. As expected, PEPC activity was higher when assayed at pH 8.1 with 2.5 mM PEP, compared with assays conducted under suboptimal conditions (pH 7.0, 0.5 mM PEP). In general, PEPC activity was about 2-fold higher in extracts from light leaves compared with dark leaves, when assays were conducted in the absence of effectors. However, a much larger difference in PEPC activity was observed when assays were conducted under suboptimal conditions in the presence of effectors. When assayed with malate, or malate plus G6P, a 20-fold difference in PEPC activity was detected (Table I). These results suggested that sensitivity of PEPC to effectors was modified by light/dark transitions of leaves.

Sensitivity of PEPC to effectors was studied further. As shown in Figure 1A, malate inhibited PEPC activity from both light and dark leaves, but the percent inhibition differed significantly. The PEPC from dark leaves was completely inhibited by 4.5 mM malate, and I_{50} (malate) was about 1.5 mM. The PEPC from light leaves was about 3-fold less sensitive to malate inhibition (Fig. 1C). Differences in G6P activation were also observed (Fig. 1B). The PEPC from both dark and light leaves was activated by G6P to about the same extent (maximum activation 3.5-fold); however, the A_{50} (G6P) was slightly higher for the dark enzyme

Table I. *Activity of PEPC Extracted from Light and Dark Maize Leaves Assayed under Different Conditions*

Assay Conditions			PEPC Activity		
pH	PEP	Additions[a]	Dark	Light	Light/Dark
	mM		*μmol/g fresh wt h^{-1}*		*ratio*
7.0	0.5	None	46	121	2.6
		G6P	111	356	3.2
		Malate	1	26	26
		Malate + G6P	9	153	17
8.1	2.5	None	240	489	2.0
		G6P	454	653	1.4
		Malate	218	450	2.1
		Malate + G6P	431	640	1.5

[a] Effectors at 5 mM in the standard reaction mixture.

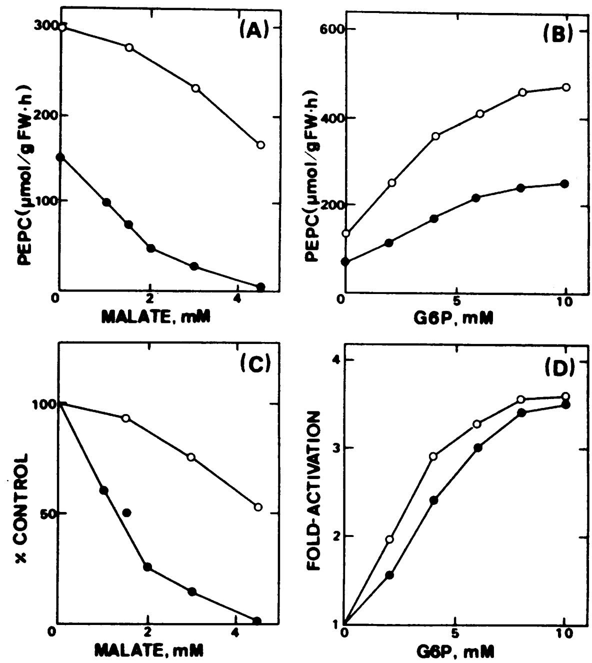

FIG. 1. Changes in sensitivity to effectors of PEPC extracted from dark (●) and light (○) leaves. Assays were all conducted at pH 7.0 with 2.5 mM PEP, and other additions as indicated. A, Malate inhibition in the presence of 5 mM G6P; B, G6P activation; C, replot of data in A as percent inhibition; D, replot of data in B as percent activation.

compared to the light enzyme (5.3 and 3.5 mM, respectively; Fig. 1D).

PEPC Protein in Leaf Extracts. Single radial immunodiffusion was used to quantitate the amount of PEPC protein in extracts from light and dark leaves. In two experiments, the concentration of PEPC protein was found to be slightly higher in extracts of light leaves; however, the difference was small (about 11%) and within experimental error. The concentration of PEPC protein was calculated to be 0.38 and 0.34 mg g^{-1} fresh weight in extracts of light and dark leaves, respectively.

The fact that PEPC protein remains essentially constant, but enzyme activity is increased by a factor of 2 to 20 (in the absence and presence of effectors, respectively, Table I) strongly suggests that PEPC activity is subjected to some posttranslational modification during light/dark transitions. The light-dark difference in relative sensitivity to effectors (Fig. 1) also strongly supports this postulate.

Effect of PEG on PEPC Activity. We examined the influence of PEG on activity of purified PEPC to determine whether subunit interactions and/or association-dissociation phenomena may influence the observed properties. Consequently, PEPC assays were conducted in the standard reaction mixture (containing H_2O alone) or in mixtures supplemented with 10% (w/v) PEG-6000 plus 0.15 M KCl. Inclusion of PEG plus KCl in the assay mixture had a substantial effect on both activity and malate inhibition of purified PEPC. As shown in Figure 2A, activity of purified PEPC in the standard reaction mixture (pH 7.0) was markedly nonlinear with PEPC concentration; activity was substantially reduced when the concentration of PEPC was below about 0.7 μg ml^{-1}. The effect of PEG plus KCl in the reaction mixture was to increase PEPC activity and essentially eliminate the discontinuity with respect to protein concentration (Fig. 2A). Under optimal assay conditions (pH 8.1), PEPC activity was strictly linear with protein concentration and PEG plus KCl had no effect (data not shown). Purified PEPC was also inhibited by malate, and PEG plus KCl decreased malate inhibition of PEPC about 3-fold (Fig. 2B).

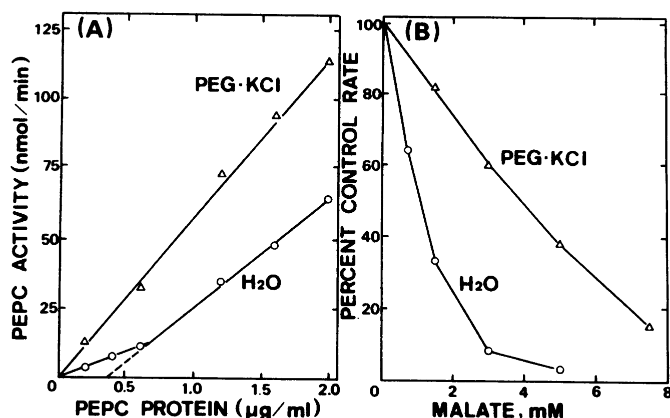

FIG. 2. Influence of PEG on (A) protein concentration dependence and (B) inhibition by malate of purified PEPC. Reaction mixtures contained 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1.25 mM PEP, 5 mM G6P, either H_2O or 10% (w/v) PEG-6000 plus 0.15 M KCl, as indicated, and in (B) 5 mM malate. Control activities in (B) were 183 and 140 nmol product/min in PEG-KCl and H_2O, respectively.

Table II. *Effect of PEG plus KCl on the Activity of PEPC Extracted from Light and Dark Maize Leaves*

Reaction[a]		PEPC Activity		
Medium	Additions	Dark	Light	Light/Dark
		µmol/g fresh wt h^{-1}		*ratio*
H_2O	None	12	35	2.9
	G6P	192	300	1.6
	G6P + malate	19	145	7.6
PEG + KCl	None	19	29	1.5
	G6P	260	320	1.2
	G6P + malate	163	237	1.5

[a] The basic mixture contained 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, and additions as indicated, in H_2O or 10% (w/v) PEG-6000 plus 0.15 M KCl. All assays contained 1.25 mM PEP. Effector concentration was 5 mM.

Subsequent experiments examined the influence of PEG plus KCl on PEPC activity in extracts from light and dark leaves. PEG plus KCl had relatively little effect on PEPC activity from light leaves, assayed in the absence of malate, but substantially increased activity from dark leaves (35 to 60%) (Table II). However, malate inhibition of both enzyme preparations was greatly reduced by PEG plus KCl (Table II). With PEPC from dark leaves, 5 mM malate caused 90% inhibition in the standard assay mixture and only 37% in PEG plus KCl. Similarly, malate inhibition of PEPC from light leaves was reduced from 51% to 26% by PEG plus KCl.

DISCUSSION

The results obtained in the present study document that PEPC in maize leaves is subjected to some form of light modulation. Dark-light transitions result in changes in PEPC activity and sensitivity to effectors. Because the amount of PEPC protein remains constant under these conditions, it is clear that some type of postranslational modification mechanism is involved. Previously, Manetas *et al.* (8) reported that light-dark transitions of *Salsola soda* PEPC resulted in changes in the affinity of the enzyme for PEP; the dark enzyme had a higher apparent K_m (PEP) and displayed positive cooperativity whereas the light enzyme had hyperbolic kinetics. Because malate is a competitive inhibitor with respect to PEP (4), it is possible that the changes in effector sensitivity observed in the present study are a manifestation of an underlying change in affinity for PEP. Since substrates and effectors are thought to bind to the enzyme at distinct sites (10), it is possible that the change in effector sensitivity cannot be entirely explained by differences in affinity for PEP. This seems likely because differences in malate inhibition were observed even in the presence of a nearly saturating level of G6P (Fig. 1), which lowers the K_m (PEP) (4, 10).

The PEPC from a number of C_4 species has been reported to contain essential and accesible sulfhydryl groups (7, 14); however, it is interesting to note that Hatch and Oliver (3) found no effect of a variety of sulfhydryl reagents on maize PEPC. Thus, some controversy remains concerning the exact role of sulfhydryl groups. However, assuming that the PEPC molecule contains sulfhydryl groups essential for catalysis and/or effector action, it is tempting to speculate that redox of these groups may be responsible for the light modulation observed here. At the present time, there is no positive evidence to support this mechanism. If sulfhydryl redox changes were responsible for the apparent light modulation of PEPC, it would be expected that the dark form of the enzyme could be activated by reductants. In preliminary experiments, this was not observed (data not shown); however, negative results cannot eliminate this possibility.

Although the exact mechanism for light modulation is unknown, it appears to involve changes in subunit interactions. This is suggested by the observation that PEG activates the dark form of the enzyme to a much greater extent than the light form (Table II). Since PEG promotes enzyme and/or subunit interactions by a 'water-exclusion' mechanism and it thereby mimics the effect of increasing protein concentration (9). Such effects of PEG on kinetic properties of phosphofructokinase have been well studied (1, 11). It is also noteworthy that PEG reduces malate inhibition of PEPC in both crude extracts and purified preparations. We postulate that malate inhibition may involve either subunit dissociation or 'loosening' of the quaternary structure of the tetrameric enzyme molecule. Further studies will be required to resolve this point. However, it is clear that a subtle change in subunit interactions and/or quaternary structure can influence both PEPC activity as well as malate inhibition, and this may be the basis for the light modulation observed.

Recent studies have also identified light modulation of sucrose phosphate synthase in maize leaves (12; SC Huber, H Usuda, W Kalt-Torres, unpublished data). Sucrose phosphate synthase and PEPC are both localized in the mesophyll cell cytoplasm in maize, and thus it is possible that both enzymes are modulated by light via a similar mechanism. At the present time, the only similarity is that neither enzyme appears to respond to redox state of sulfhydryl groups. Hence, it appears that light regulates the activity of two cytoplasmic enzymes in maize leaves that are involved in primary (PEPC) and secondary (sucrose-P synthase) carbon metabolism. Work is underway to elucidate the mechanism(s) involved.

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