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Regulation of Phosphoenolpyruvate Carboxylase Activity in Maize Leaves[1]

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ABSTRACT

The aim of this work was to investigate how light regulates the activity of phosphoenolpyruvate carboxylase *in vivo* in C_4 plants. The properties of phosphoenolpyruvate carboxylase were investigated in extracts which were rapidly prepared (in less than 30 seconds) from darkened and illuminated leaves of *Zea mays*. Illumination resulted in a significant decrease in the $S_{0.5}$(phosphoenolpyruvate) but there was no change in V_{max}. The form of the enzyme from illuminated leaves was less sensitive to malate inhibition than was the form from darkened leaves. At low concentrations of phosphoenolpyruvate, the activity of the enzyme was strongly stimulated by glucose-6-phosphate, fructose-6-phosphate, triose-phosphate, alanine, serine, and glycine and was inhibited by organic acids. The enzyme was assayed in mixtures of metabolites at concentrations believed to be present in the mesophyll cytosol in the light and in the dark. It displayed low activity in a simulated 'dark' cytosol and high activity in a simulated 'light' cytosol, but activities were different for the enzyme from darkened compared to illuminated leaves.

PEP[2] carboxylase occurs widely, perhaps universally, in cells of higher plants (11), but in plants with C_4 and CAM modes of photosynthesis, specific isoenzymes are present in the cytosol of photosynthetic tissues at very much higher activities than in C_3 photosynthetic or in nonphotosynthetic tissues (11, 26). In C_4 plants, PEP carboxylase is the initial step of the 'CO_2 pump' which transfers CO_2 from mesophyll cells and concentrates it in the bundle-sheath cells. Not only must the CO_2 pump be coordinated with the rate of Calvin cycle turnover, but PEP consumption is also likely to be regulated so that photosynthesis can proceed efficiently at different irradiances, temperatures, and CO_2 concentrations, and particularly to ensure that glycerate-3-P is not drained into the C_4 cycle during photosynthesis (6). Since PEP also lies at an important branch-point in plant metabolism its utilization by PEP carboxylase must also be curtailed in darkness.

There is good evidence that light modulation of PEP carboxylase occurs *in vivo*. For example, PEP has been observed to increase transiently upon illumination of maize leaves (6) and the steady state PEP content of leaves of maize and of *Amaranthus edulis* is relatively stable in relation to irradiance (RC Leegood, S von Caemmerer, unpublished results). However, it is not clear how regulation of PEP carboxylase occurs in C_4 plants. Activation in the light could be achieved by, for example, covalent modification (*e.g.* phosphorylation [2]) or the enzyme could be regulated by metabolites whose levels change upon illumination. Since PEP carboxylase is a cytosolic enzyme it is unlikely to be controlled in the same way by light as are the chloroplastic enzymes, although factors such as the pH of the cytosol might change in response to light (18). In CAM plants regulation by light may occur through a dimer-tetramer conversion, possibly mediated by Mg^{2+} (30), or by phosphorylation of the enzyme (19), with the result that the active enzyme from darkened leaves has a lower K_m (PEP) and is rendered much less sensitive to inhibition by malate (19, 29, 30).

The evidence for regulation of the enzyme directly by light in C_4 plants has, until recently, been weak. There have been reports of 2-fold changes in V_{max} and a reduction in K_m, but these changes vary between species (9, 10). These reports can be criticized on the grounds that the extraction procedure lasted 15 min and that any light-induced changes might by then have been reversed. However, Huber and Sugiyama (8) have recently reported that a change in the sensitivity of PEP carboxylase to effectors occurs during light-dark transitions. The evidence for metabolite modulation of PEP carboxylase is altogether stronger. Unlike PEP carboxylases from C_3 and CAM plants, PEP carboxylase from C_4 plants has a relatively low affinity for PEP (25, 26), but in common with them, the enzyme from C_4 plants is inhibited by organic acids such as malate and aspartate and, like the enzyme from CAM plants, is also strongly activated by glucose-6-P (4, 7, 24, 27). With recent information on the concentrations of metabolites in the mesophyll cells of maize leaves (12, 13, 22, 23) it is now possible to estimate the effects of physiological concentrations of various metabolites on the enzyme. In this paper we report studies both of light-activation of the enzyme in leaves and of the sensitivity of the enzyme to metabolite effectors in order to estimate how the enzyme behaves *in vivo*.

MATERIALS AND METHODS

Plant Material. *Zea mays* L., var Kelvedon Glory, was grown in a glasshouse between October 1985 and October 1986 in a mixture of compost and vermiculite, with additional artificial illumination for 9 h/d. The third leaves from 3-week-old plants were taken, the midrib removed, and portions of the central part of the leaf were cut into 2-cm-long pieces and placed on damp filter paper. They were darkened for 20 h and were subsequently illuminated at 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR) for 15 min. Periods of illumination of leaves for longer than 15 min did not result in further observable changes in the properties of the enzyme.

Extraction and Assay of PEP Carboxylase. Leaf sections were rapidly homogenised in a glass-in-glass homogeniser (<30 s) in 1 ml of 50 mM Mops-KOH (pH 7.2), 10 mM $MgCl_2$, 1 mM DTT,

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[2] Abbreviations: PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; $S_{0.5}$, substrate concentration yielding half-maximal rate.

25% (v/v) glycerol, and 2% (w/v) BSA, and 50 μl aliquots were added to 450 μl of assay medium to give final concentrations of 90 mM Tricine-HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 mM NADH, 10 mM $NaH^{14}CO_3$ (1.3 TBq·mol^{-1}), 0.5 to 10 mM PEP, and 2.7 units malate dehydrogenase. After 30 s the reactions were stopped with 450 μl 10 M HCOOH, the mixtures taken to dryness, then a further 100 μl HCOOH were added and the mixtures dried down again. The ^{14}C fixed was determined by liquid scintillation counting.

For the continuous spectrophotometric assay, the leaf extract was centrifuged in an Eppendorf centrifuge 5414 for 20 s and 50 μl of the supernatant fluid were added to 950 μl of the assay medium described above with the omission of radioactive bicarbonate. The change of absorbance was followed at 340 nm for up to 5 min.

The radiometric assay was linear over the first 30 s both in the absence and presence of metabolites, with the exception of glucose-6-P for which a short activation lag was observed. There was therefore good agreement between data obtained by radiometric and spectrophotometric means. Introduction of a preincubation step by incubating the extract with the added metabolite for 2 min before adding $NaH^{14}CO_3$ did not alter the observed results for $S_{0.5}$(PEP) or V_{max}.

Inclusion of 10 mM fluoride (to prevent the possible action of phosphatases on the PEP carboxylase protein) in the extraction medium did not influence the degree of light-activation observed. Changes in the extraction pH, and the inclusion of various thiols in the extraction and assay media (*e.g.* DTT, mercaptoethanol) also had no appreciable influence. Recently it has been reported that nonenzymatic reactions of the product (the keto form of oxaloacetate) can lead to underestimation of the activity of the enzyme (28). However a Pi-release assay was not employed routinely in these studies due to nonspecific release of Pi in crude extracts. Kinetic parameters were determined by direct linear plots, when appropriate, by use of a computer program (ENZPACK, Elsevier-Biosoft, Cambridge, U.K.).

Chlorophyll. Chl was determined as described by Bruinsma (1).

Glucose-6-P Measurement. Glucose-6-P was determined as described by Lowry and Passonneau (16) after addition of 50 μl 10 M formic acid to the assay sample and neutralisation with Na_2CO_3.

RESULTS AND DISCUSSION

Changes in the Kinetic Properties of PEP Carboxylase following Illumination of Leaves. PEP carboxylase was rapidly extracted from darkened and illuminated maize leaves and immediately assayed, and the $S_{0.5}$(PEP) and V_{max} were estimated. The standard rapid assay involved incubation of the crude extract in a reaction mixture for a period of 30 s. Most assays reported in this paper were done at pH 7.5, since estimates of the pH of the cytosol *in vivo* range between pH 7 and pH 8 (5, 18). The pH of the cytosol is unlikely to be as high as the pH optimum of PEP carboxylase (pH 8.0–8.5, although see Ref. 28), or as low as pH 7.0, at which pH PEP carboxylase displays low activities which would be incapable to supporting observed rates of photosynthesis. The data in Table I show that when PEP carboxylase was extracted from illuminated as compared to darkened leaves, $S_{0.5}$ decreased while V_{max} remained constant. Light-activation did not therefore result in a change in V_{max} as reported for the C_4 plant *Setaria verticillata* (10). After rapid extraction by this procedure, PEP carboxylase displayed hyperbolic or very slightly sigmoidal kinetics with respect to PEP at the pH employed (Fig. 1) with values for $S_{0.5}$ which were comparable to other values reported for maize leaf PEP carboxylase (25, 27).

Influence of Metabolites on the Activity of PEP Carboxylase. Purified PEP carboxylase from leaves of C_4 plants is known to be modulated by a number of metabolites, including glucose-6-P and glycine, which activate it strongly (4, 7, 20, 24, 27), and organic acids such as malate and aspartate, which inhibit it strongly (7, 15, 24). The effects of these and other metabolites were tested using both the rapid radiometric assay and the spectrophotometric assay. The data in Table II show how metabolites affected the affinity for PEP, measured either by the change in $S_{0.5}$ or by their effect on the rate at a limiting concentration (0.5 mM) of PEP. Although the rapid assay involved carryover of metabolites, these would have been present at such low dilution (*e.g.* the maximum concentration of malate would have been approximately 20 μM) that their effect on the activity of the enzyme would have been negligible. Similarly, interconversion of added effectors would have been minimal during the rapid assay. We checked that the stimulation by fructose-6-P was not due to conversion of fructose-6-P to glucose-6-P by phosphohexose isomerase present in the crude extract. When 2 mM fructose-6-P was incubated with the leaf extract the concentration of glucose-6-P rose to 20 μM after 30 s. At this low concentration, added glucose-6-P did not perceptibly stimulate PEP carboxylase activity (half-maximal activation by glucose-6-P, measured in the presence of 3 mM PEP, was observed at a concentration of 1 mM).

Triose-P, serine, alanine, and fructose-6-P showed much larger effects on the enzyme (Table II) than have previously been reported (3, 15, 20), indicating that the regulatory response of the enzyme may be modified during purification. Triose-P (2 mM) has been reported to be an activator at low (<2 mM)

Table I. *Light Activation of PEP Carboxylase in Maize Leaves*

Properties of PEP carboxylase from darkened and illuminated maize leaves. The enzyme was rapidly extracted and assayed by incorporation of $H^{14}CO_3^-$ over a period of 30 s. Data shown are means ± SE with the number of samples in parentheses. The test of significance (P) was made by analysis of variance.

Leaf Treatment	$S_{0.5}$(PEP)	V_{max}
	mM	*μmol·h^{-1}·mg^{-1} Chl*
Dark	2.25 ± 0.18 (16)	494 ± 90 (13)
Light	1.60 ± 0.13 (16)	416 ± 59 (13)
P	<0.01	NS[a]

[a] Not significant.

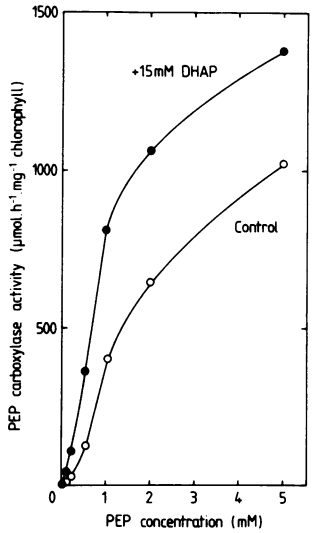

FIG. 1. Effect of 15 mM DHAP on the activity of PEP carboxylase extracted from illuminated leaves; the rate was measured by the radiometric assay with varying [PEP]. The concentration of Mg^{2+} was 10 mM.

Table II. *Modulation of PEP Carboxylase Activity by Metabolite Effectors*

The enzyme was rapidly extracted from illuminated leaves and $S_{0.5}$ was determined in a rapid radiometric assay; effects on the velocity at limiting PEP were determined in a spectrophotometric assay. The mean $S_{0.5}$ for control was 1.66 mM.

Effector	Effect on Velocity at 0.5 mM PEP	Apparent $S_{0.5}$(PEP), Mean
mM	*% of control rate*	*mM*
3 mM glucose-6-P	417 (3)[a]	0.14 (3)
2 mM fructose-6-P	283 (4)	0.77 (3)
5 mM triose-P	299 (4)	0.82 (3)
15 mM triose-P	394 (4)	0.65 (3)
0.1 mM fructose-2,6-bisP	119 (4)	ND[b]
1 mM fructose-1,6-bisP	87 (4)	ND
0.3 mM glucose-1-P	111 (4)	ND
0.8 mM UDP-glucose	100 (4)	ND
3 mM glycerate-3-P	85 (9)	ND
1 mM glycerate-2-P	84 (4)	ND
1 mM pyruvate	125 (3)	ND
0.1 mM pyrophosphate	117 (4)	ND
5 mM phosphate	59 (4)	ND
5 mM serine	370 (4)	1.08 (3)
10 mM glycine	314 (4)	0.70 (2)
5 mM alanine	206 (5)	1.11 (3)
5 mM lysine	330 (6)	ND
5 mM proline	159 (6)	ND
5 mM cysteine	161 (2)	ND
5 mM threonine	145 (4)	ND
5 mM glutamine	144 (5)	ND
5 mM arginine	140 (2)	ND
2 mM malate	34 (13)	>3
20 mM glutamate	10 (4)	>5
5 mM 2-oxoglutarate	30 (3)	ND
5 mM aspartate	10 (3)	ND

[a] Number of samples in parentheses. [b] Not determined.

concentrations of PEP and an inhibitor at higher (5–10 mM) concentrations of PEP (3). In the present studies, 2 mM triose-P had a relatively weak effect on enzyme activity, but at higher concentrations it activated the enzyme strongly at all concentrations of PEP (Fig. 1; Table II). Half-maximal activation was observed at a concentration of 10 mM DHAP in the presence of 3 mM PEP.

Phosphate was an inhibitor of PEP carboxylase (Table II), but the inhibition was greater than previously reported (25, 27). This may be of importance *in vivo* since Pi is a key metabolite in photosynthetic cells and there is almost certainly a concentration gradient of Pi between mesophyll and bundle-sheath. Its concentration may therefore be expected to change during photosynthesis and following illumination (Table V).

Glycerate-3-P had little effect on enzyme activity either at pH 7.5 (Table II) or at pH 7.0 (results not shown), although other workers have reported a stimulation by 3-PGA at pH 7.2 and an inhibition at pH 7.8 to 8.0 (3, 21). In agreement with earlier studies, it was found that pyruvate had little effect on the activity of the enzyme (15, 25), but stimulation by alanine was much larger than previously reported (20). A number of other amino acids stimulated enzyme activity (Table II). Stimulation of activity by amino acids may therefore be relatively nonspecific, although homoserine, asparagine and methylamine, all added at a concentration of 5 mM, were without any influence on the activity of the enzyme.

The results in Table III show that light activation of PEP carboxylase in maize leaves was accompanied by a decrease in the sensitivity to inhibition by malate. In this instance, light activation in the control was evident by an increase in activity

Table III. *Sensitivity of PEP Carboxylase to Malate Inhibition*

Darkened or illuminated maize leaves were rapidly extracted, and the effect of 1 mM malate on the activity of the enzyme at 0.5 mM PEP, 10 mM Mg^{2+}, was determined in a rapid radiometric assay. The data shown are means ± SE of six separate samples. The test of significance (P) was made by analysis of variance.

Leaf Treatment	Activity		Inhibition by Malate
	Control	+1 mM malate	
	$\mu mol \cdot h^{-1} \cdot mg^{-1}$ *Chl*		%
Dark	53 ± 5	6 ± 0.6	89 ± 1
Light	109 ± 9	38 ± 5	65 ± 3
P	<0.001	<0.001	<0.001

at limiting PEP (*cf.* 8). When the activity of the enzyme was measured in the presence of a range of malate concentrations (Fig. 2), it was found that inhibition by malate was more marked with the enzyme extracted from darkened than from illuminated leaves, particularly in the physiological range of malate concentrations, *i.e.* 10 to 30 mM. At very low concentrations of malate there was often a slight stimulation of activity of the enzyme extracted from illuminated leaves. Half-maximal inhibition by malate in the presence of 3 mM PEP increased from 4.5 to 10 mM following illumination of leaves. This has also been shown by Huber and Sugiyama (8) and further demonstrates that light activation is accompanied by a change in the properties of the enzyme. This change in the sensitivity of the enzyme to inhibition by malate may be one means by which the effect of the increase in malate observed upon illumination is overcome. Dark

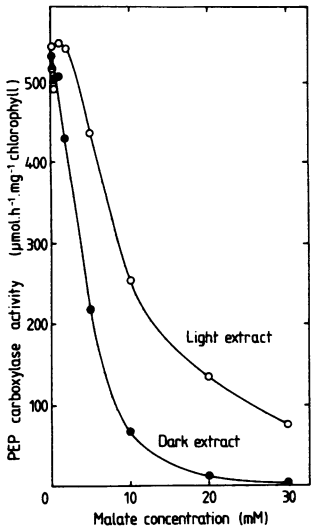

FIG. 2. Effect of malate concentration on the activity of PEP carboxylase extracted from darkened and illuminated leaves. The concentration of PEP in the radiometric assay was 3 mM, and Mg^{2+} was 10 mM. Note that malate inhibition was less severe here than in Table III, because [PEP] was higher.

activation of PEP carboxylase in leaves of CAM plants is also accompanied by a decrease in the sensitivity of the enzyme to inhibition by malate and by an increase in the affinity for PEP (19, 29, and references cited therein). In CAM plants, the enzyme is therefore more active in darkened leaves (in which the malate content is initially low) and less active in illuminated leaves (in which the malate levels are initially high).

Modulation of PEP Carboxylase Activity by Changes in Metabolites during Photosynthesis. PEP carboxylase is evidently subject to control by a large number of metabolites of physiological relevance. Of particular importance are two metabolites which are known to build up during photosynthesis in the mesophyll cells in order to power diffusive transport to the bundle-sheath cells. These metabolites comprise the inhibitor, malate, and the activator, triose-P. Malate and triose-P will be present in the mesophyll cytosol of maize at concentrations which will exceed 10 mM when rates of photosynthesis are high (13, 22, 23). Since malate at these concentrations is a potent inhibitor even of the light-activated form of PEP carboxylase (Fig. 2), this poses the question as to which compounds are physiologically important in reversing malate inhibition *in vivo*. Huber and Edwards (7) and Ting and Osmond (24) have shown that glucose-6-P can reverse the inhibition by malate of PEP carboxylase from C_4 and CAM plants, but the role of triose-P has not been studied. The amounts of hexose-P and PEP rise during illumination of maize leaves and appreciable portions of the pools of these metabolites may be expected to be present in the mesophyll cytosol (13, 22, 23). For example, in maize leaves, the content of glucose 6-P is less than 10 nmol·mg^{-1} Chl in darkness and rises to between 100 and 200 nmol·mg^{-1} Chl in the light. The content of triose-P is generally less than 30 nmol·mg^{-1} Chl in darkness and rises to between 500 and 1000 nmol·mg^{-1} Chl in the light. PEP contents in the dark are variable, being less than 5 nmol·mg^{-1} Chl after short dark periods (*e.g.* 20 min) but 50 to 70 nmol·mg^{-1} Chl after several hours darkness. In the light the PEP content of leaves is usually in the range of 100 to 150 nmol·mg^{-1} Chl (6, 13, 14, 22, 23). If the cytosolic volume is equivalent to 25 $\mu l \cdot mg^{-1}$ total leaf Chl, then 100 nmol·mg^{-1} Chl is equivalent to a concentration of 4 mM. In the following simulations the lowest estimates for PEP concentration have been employed for the 'dark cytosol' (however, these can be adjusted by reference to Fig. 3).

Physiological concentrations (13, 22, 23) of regulatory metabolites were included in assay mixtures to assess the extent to which PEP carboxylase activity might be expected to be affected by the mesophyll cytosolic environment thought to exist *in vivo*. The total concentration of Mg^{2+} in the assay was increased in some of these experiments to 30 mM in order to compensate for binding of Mg^{2+} by effector metabolites. Triose-P overcame the inhibition of the enzyme by malate when added in equimolar amounts (Table IV). The simplest simulated cytosol which was employed contained PEP, malate and triose-P (Table V). There was a dramatic effect of these mixtures of metabolites on the activity of the enzyme, whether it was extracted from darkened or illuminated leaves. The difference in activity between the enzyme extracted from illuminated and darkened leaves and assayed in the dark cytosol may largely reflect the change in malate sensitivity (Fig. 2), while in the 'light cytosol' the variation in light activation was large and there were often greater differences than indicated in Table V (Fig. 3).

A number of more complex assay media were tested, including

Table IV. *Relief of Malate Inhibition of PEP Carboxylase by DHAP*

Illuminated maize leaves were rapidly extracted and assayed in the radiometric assay in the presence of added metabolites. The concentration of PEP was 3 mM and Mg^{2+} was 30 mM.

Addition	Activity
	$\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl
A. None	336
+ 2 mM malate	266
+ 2 mM DHAP	406
+ 2 mM malate, + 2 mM DHAP	330
B. None	368
+ 20 mM malate	188
+ 20 mM DHAP	515
+ 20 mM malate, + 20 mM DHAP	322

Table V. *Activity of PEP Carboxylase in Simulated Cytosolic Environments*

Darkened or illuminated maize leaves were rapidly extracted and assayed in a rapid radiometric assay in the presence of the metabolites shown. The concentration of Mg^{2+} was 30 mM. Data shown are means ± SE for three samples.

Components of Assay Medium	Activity	
	Dark extract	Light extract
	$\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl	
A. Dark (0.1 mM PEP, 5 mM malate, 0.5 mM DHAP)	12 ± 1	26 ± 6
Light (3 mM PEP, 20 mM malate, 15 mM DHAP)	377 ± 85	402 ± 61
B. Dark (0.1 mM PEP, 5 mM malate, 0.5 mM DHAP, 0.1 mM glucose-6-P)	16 ± 3	22 ± 3
Light (3 mM PEP, 20 mM malate, 15 mM DHAP, 3 mM glucose-6-P)	522 ± 27	645 ± 31
C. Dark (0.1 mM PEP, 5 mM malate, 0.5 mM DHAP, 0.1 mM glucose-6-P, 0.05 mM fructose-6-P, 25 mM Pi, 1 mM glycerate-3-P)	9 ± 1	9 ± 1
Light (3 mM PEP, 20 mM malate, 15 mM DHAP, 3 mM glucose-6-P, 1.5 mM fructose-6-P, 5 mM Pi, 5 mM glycerate-3-P)	319 ± 47	352 ± 38

additional metabolites such as glucose-6-P, fructose-6-P, alanine, aspartate, glycerate-3-P, and Pi (in the latter case it was assumed that [Pi] was 5 mM in the light cytosol and the decreased amounts of phosphorylated intermediates in the dark cytosol were matched by a corresponding increase in the amount of Pi). These more complex simulated cytosolic environments, one of which is shown in Table V, did not bring about an appreciable departure from the pattern observed in the simple simulated cytosol. The total activities were of the same order of magnitude while the degree of observed light activation varied slightly. It should be noted that the combination of metabolites present in the light cytosol could increase enzyme activity by between 20- and 50-fold compared with the dark cytosol, quite independently of light activation.

Figure 3 amplifies the data for the simplest simulated cytosol in Table V by showing the combined influence of light-induced changes in the amounts of malate and triose-P and of changes in PEP concentration on the activity of the enzyme at both high and low [Mg^{2+}]. Although the response was different at the two concentrations of Mg^{2+}, the following general features were evident: (a) the components of the light cytosol inhibited the activity of the enzyme extracted from darkened leaves, particularly at 10 mM Mg^{2+}, while, by comparison, the light cytosol was less inhibitory to the enzyme extracted from illuminated leaves. (b) At 10 mM Mg^{2+} the activity of the enzyme extracted from illuminated leaves in the light cytosol was less than the activity in the dark cytosol for a given concentration of PEP, but at 30 mM Mg^{2+} this pattern was reversed and the enzyme extracted from illuminated leaves was more active in the light cytosol than in the dark cytosol. (c) The response to PEP was strongly sigmoidal when the enzyme from darkened leaves was assayed in the light cytosol (*i.e.* in the presence of large amounts of malate) but was less sigmoidal in the dark cytosol or when the enzyme from illuminated leaves was assayed in either simulated cytosol. Addition of glucose-6-P also removed sigmoidicity with respect to [PEP] in the light cytosol mixture (results not shown).

To estimate the concentration of free Mg^{2+} present in these experiments, Mg^{2+}-binding constants for these metabolites were determined under standard assay conditions at pH 7.5 by the method of Manchester (17). Binding constants, K′, were 315, 57, 226, and 48 M^{-1} for PEP, malate, DHAP, and glucose-6-P, respectively (where $K' = [MgL]/[Mg^{2+}]([L]-[MgL])$; L = total amount of the binding ligand, Mg^{2+} = concentration of free Mg^{2+}). In the light cytosol (3 mM PEP, 15 mM DHAP, 20 mM malate) the concentrations of free Mg^{2+} determined by this method were 0.6 and 3.7 mM in the presence of 10 and 30 mM Mg^{2+}, respectively.

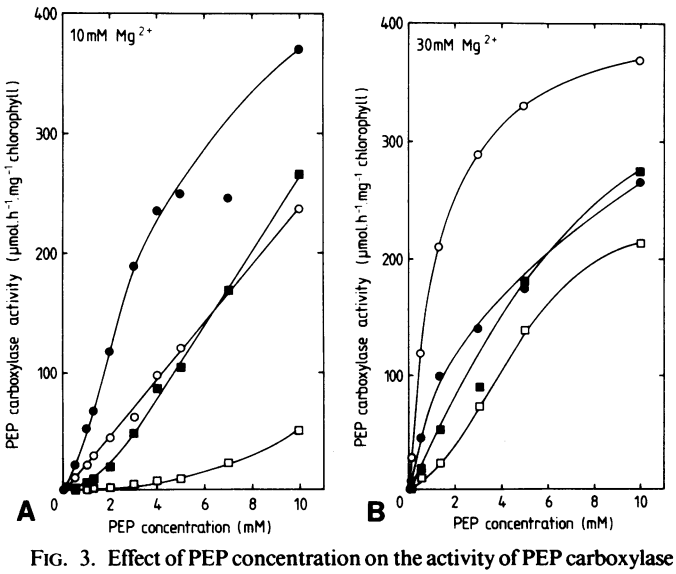

FIG. 3. Effect of PEP concentration on the activity of PEP carboxylase extracted from darkened and illuminated leaves and measured using the radiometric assay in simulated dark cytosol (5 mM malate, 0.5 mM DHAP) or light cytosol (20 mM malate, 15 mM DHAP). The Mg^{2+} concentration in the assay was 10 mM (A) or 30 mM (B). Dark extract: dark cytosol (■), light cytosol (□); light extract: dark cytosol (●), light cytosol (○).

Light activation (expressed as the ratio of activities of the enzyme from darkened and illuminated leaf tissue measured in the light cytosol at a particular [PEP]) was therefore evident at both [Mg^{2+}]. The combination of a light-induced change in sensitivity of PEP carboxylase to malate and the increased affinity for PEP resulted in a dramatic increase in activity and a large change in the apparent affinity for PEP. An important feature of these results is that under simulated cytosolic conditions, regardless of the concentration of Mg^{2+}, the $S_{0.5}$(PEP) was comparable to physiological concentrations of PEP. With the enzyme extracted from illuminated leaves and assayed in the light cytosol, the $S_{0.5}$(PEP) was about 1.3 mM with 30 mM Mg^{2+} and about 7.5 mM with 10 mM Mg^{2+}. The pattern of response was probably different at the two concentrations of Mg^{2+} because Mg^{2+} counteracts the inhibition of the enzyme by malate (7) and because stimulation by DHAP was enhanced at higher Mg^{2+} concentrations (results not shown).

Implications for the Regulation of PEP Carboxylase *in Vivo*. It is evident from the above results that light activation as such (*i.e.* changes in the sensitivity to malate, the affinity for PEP, and in the sensitivity to glucose-6-P [8]) and light-induced changes in amounts of regulatory metabolites will both be important in determining the activity of PEP carboxylase *in vivo*. The data also show that DHAP will be important in reversing malate inhibition of PEP carboxylase *in vivo* (Table IV) in addition to the well characterised relief of malate inhibition by glucose-6-P (7, 24). The response of the enzyme to PEP concentration under simulated cytosolic conditions shows that the enzyme is unlikely to be saturated with PEP and that changes in PEP concentration *in vivo* will therefore be an important influence on the activity of the enzyme.

The maximum catalytic activity of PEP carboxylase (measured in the presence of activators such as glucose-6-P) is several-fold higher than rates of carbon assimilation in maize leaves (13). On the other hand, physiological concentrations of malate strongly inhibit the enzyme. If PEP carboxylase is to play a part in the control of photosynthetic flux, then its activity must be comparable to rates of photosynthetic carbon assimilation. Accordingly, a balance must be struck between the amounts of activators and inhibitors present in the cytosol.

How might metabolite modulation of PEP carboxylase permit it to sense events occurring in the mesophyll cytosol and chloroplasts and allow coordination of the C_4 cycle with the Calvin cycle during photosynthesis? One mechanism which would allow coordination with other reactions in the C_4 cycle would be modulation by the products of carboxylation—malate and aspartate and by the substrate, PEP. It has already been seen that changes in the concentration of PEP and malate within the physiological range will be important. PEP carboxylase could also respond indirectly to the level of pyruvate because pyruvate in the cytosol is likely to be in equilibrium with alanine, which is an activator. The fact that PEP carboxylase activity is modulated by triose-P and hexose-P will be important in determining its activity in response to the supply of glycerate-3-P (which can be considered to be the metabolite 'message' from the Calvin cycle) and to the rate of triose-P utilization by sucrose synthesis in the mesophyll cytosol and to triose-P consumption in the Calvin cycle. The production of hexose-P in the mesophyll cytosol is strongly regulated by the level of fructose-2,6-bisP (22),

to which PEP carboxylase is itself unresponsive (Table II). Regulation by the fructose-2,6-bisP system ensures that sucrose synthesis is highly responsive to the supply of triose-P and that synthesis of hexose-P is triggered by an appropriate threshold concentration of triose-P. Hexose P will enhance the response of the enzyme to the supply of triose-P (Table V). In effect then, the delicately balanced regulatory properties of the sucrose synthesizing system, which recognize the rate of carbon output from the Calvin cycle, will be conferred on PEP carboxylase. Inhibition of PEP carboxylase by Pi will reinforce such regulation. The tendency for diversion of glycerate-3-P to PEP by excessive rates of PEP carboxylation will be ameliorated both by accumulation of the inhibitor, malate, and by decreases in the levels of the activators, triose-P and hexose-P. It is also important to note that PEP carboxylase is very sensitive to PEP, malate, and triose-P, the production and consumption of which is dependent upon changes in electron transport capacity within the mesophyll chloroplast. This will ensure a rapid response to, for example, lowered irradiance. In these ways rates of initial carboxylation in the C_4 cycle can be linked to the rates of C_4 cycle and Calvin cycle turnover and to rates of product synthesis, despite the complexity and compartmentation of the pathway.

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