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Metabolite Regulation of Partially Purified Soybean Nodule Phospho*enol*pyruvate Carboxylase[1]

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ABSTRACT

Phospho*enol*pyruvate carboxylase (PEPC) was purified 40-fold from soybean (*Glycine max* L. Merr.) nodules to a specific activity of 5.2 units per milligram per protein and an estimated purity of 28%. Native and subunit molecular masses were determined to be 440 and 100 kilodaltons, respectively, indicating that the enzyme is a homotetramer. The response of enzyme activity to phospho*enol*pyruvate (PEP) concentration and to various effectors was influenced by assay pH and glycerol addition to the assay. At pH 7 in the absence of glycerol, the K_m (PEP) was about twofold greater than at pH 7 in the presence of glycerol or at pH 8. At pH 7 or pH 8 the K_m (MgPEP) was found to be significantly lower than the respective K_m (PEP) values. Glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate, and dihydroxyacetone phosphate activated PEPC at pH 7 in the absence of glycerol, but had no effect under the other assay conditions. Malate, aspartate, glutamate, citrate, and 2-oxoglutarate were potent inhibitors of PEPC at pH 7 in the absence of glycerol, but their effectiveness was decreased by raising the pH to 8 and/or by adding glycerol. In contrast, 3-phosphoglycerate and 2-phosphoglycerate were less effective inhibitors at pH 7 in the absence of glycerol than under the other assay conditions. Inorganic phosphate (up to 20 millimolar) was an activator at pH 7 in the absence of glycerol but an inhibitor under the other assay conditions. The possible significance of metabolite regulation of PEPC is discussed in relation to the proposed functions of this enzyme in legume nodule metabolism.

The anaplerotic fixation of CO_2 by PEPC[3] (EC 4.1.1.31) is believed to play a variety of roles in support of symbiotic N_2 fixation by legume root nodules. These include: (a) the synthesis of dicarboxylic acids (malate, succinate) used as respiratory substrates by the bacteroids (10, 20), (b) provision of carbon skeletons for NH_4^+-assimilation (3, 20), and (c) synthesis of organic acids to maintain charge balance and neutral pH intracellularly and in the xylem (8). Most of the evidence for the first two functions comes from physiological studies of $[^{14}C]CO_2$ fixation by detached nodules or nodulated roots. Recently, Rosendahl *et al.* (20) examined the distribution of metabolites of dark CO_2 fixation in pea root nodules inoculated with effective and ineffective strains of *Rhizobium leguminosarum*. Their results have provided the first unequivocal demonstration that organic acids produced following nodule CO_2 fixation are taken up by the bacteroids.

Compared with the plethora of physiological data, little is known about the biochemistry of legume nodule PEPC. The enzyme has been partially purified from French bean, soybean, lupin, and alfalfa nodules (3, 6, 11, 16, 25). No regulatory metabolites have yet been identified for PEPC isolated from either soybean or alfalfa nodules (16, 25). However, potential regulators were tested at saturating PEP concentrations and this may explain the lack of any effect. An early report on lupin nodule PEPC listed Asp, isocitrate, ATP, glycerate, malate, and phosphoglycolate as inhibitors (3). More recently, Marczewski (11) resolved two isoforms of PEPC from lupin nodules and showed that they were inhibited by Asp, malate, Glu, pyruvate, and 2-OG. Assay pH influenced inhibition by malate and Asp with Asp being inhibitory at pH 7.2, but not at pH 8, and malate being a stronger inhibitor at pH 8 than at pH 7.2.

Assay pH is also known to affect the response of maize leaf PEPC to two effectors, malate and Glc-6-P. Malate is a stronger inhibitor and Glc-6-P a stronger activator at pH 7 than at pH 8 (18, 27). Raising the pH from 7 to 8 or adding glycerol promotes conversion of dilute solutions of maize leaf PEPC from its dimeric to its native tetrameric form and substantially reduces or eliminates malate inhibition (18, 27).

In the present study we examine metabolite regulation of partially purified soybean nodule PEPC at subsaturating concentrations of PEP. We also examine the effects of assay pH and glycerol addition to the assay. Finally, we use these results to formulate a model for the regulation of PEPC in relation to the proposed functions of this enzyme in nodule metabolism.

MATERIALS AND METHODS

Plant Material and Bacterial Strain

Soybean (*Glycine max* L. Merr. cv Maple Arrow) plants nodulated with *Bradyrhizobium japonicum* strain USDA 16 were grown in silica sand in a naturally illuminated greenhouse with supplemental lighting as previously described (10). Nodules were harvested 4 to 6 weeks after planting, immediately frozen in liquid N_2, and then stored at −70°C until used in the purification of PEPC.

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[3] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; PEP, phospho*enol*pyruvate; GS, glutamine synthetase; GOGAT, glutamine-oxoglutarate aminotransferase; DHAP, dihydroxyacetone phosphate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; 2-OG, 2-oxoglutarate.

PEPC Assay

For routine measurements of enzyme activity, the PEPC reaction was coupled with the malate dehydrogenase reaction and assayed at 25°C by monitoring NADH oxidation at 340 nm using a Varian DMS 200 spectrophotometer. The standard PEPC assay contained 25 mM Bis-Tris-Propane (pH 8), 15% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM $KHCO_3$, 5 mM DTT, 0.2 mM NADH, 2.5 mM PEP, and 10 units of malate dehydrogenase in a final volume of 1 mL. Assays were initiated by the addition of the enzyme preparation. One unit of enzyme activity is defined as the amount catalysing the oxidation of 1 μmol NADH·min^{-1} at 25°C.

Kinetic Studies

Kinetic analyses and tests of potential metabolite effectors were performed in a modification of the standard assay. Hepes (100 mM) replaced Bis-Tris-Propane. In some experiments glycerol was omitted and/or the pH was adjusted to 7 (see legends to Tables). The four alternative assay conditions used were: (a) pH 7, no glycerol, (b) pH 8, no glycerol, (c) pH 7, with 15% (v/v) glycerol, and (d) pH 8, with 15% (v/v) glycerol. K_m (PEP) and V_{max} were determined using Enzfitter (Sigma Chemical Co.), a nonlinear regression program. Activation constants (K_a) were determined using double reciprocal plots of $1/(v\text{-}v_0)$ *versus* 1/[activator], where v and v_0 represent the activity in the presence and absence of activator, respectively. I_{50} values (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job *et al.* (9). All kinetic data are the means of duplicate determinations performed on two separate enzyme preparations and are reproducible to within ±10% SE.

Calculation of the Concentration of Mg Complexes

The concentration of the Mg complexes with Pi or PEP in the PEPC assay was calculated using:

$$C = ([K_d + M + S) - [(K_d + M + S]^2 - 4\ MS]^{0.5})/2,$$

where C = concentration of the complex, M = total Mg^{2+} concentration, S = total Pi or PEP concentration and K_d = the apparent dissociation constant of the complex defined by the relationship K_d = ([free S] × [free M])/[C] (2). The absolute dissociation constants for MgPi and MgPEP (5) were corrected for pH.

Protein Assay

Protein concentration was determined according to the method of Bradford (1) using bovine γ-globulin as standard.

Other Enzyme Assays

Hexose-P isomerase (4), aldolase (15), and PEP phosphatase (7) were assayed as previously described. PGA mutase was assayed in a mixture containing 100 mM Hepes-NaOH (pH 7.2), 10 mM $MgCl_2$, 0.05 mM NADH, 2.7 mM ADP, 1 unit enolase, 5 units pyruvate kinase, 6 units lactate dehydrogenase, and 3 mM 3-PGA in a final volume of 1 mL. Enolase was assayed as described for PGA mutase except that the enolase coupling enzyme was omitted and 0.5 mM 2-PGA replaced 3-PGA. Triose-P isomerase was assayed in a mixture containing 20 mM Tris-acetate (pH 7.2), 2 mM sodium arsenate, 0.06 mM NAD, 3 units glyceraldehyde 3-P dehydrogenase, and 2.5 mM DHAP.

Buffers Used in PEPC Purification and Analytical Superose 6 Gel Filtration

Buffer A: 25 mM KPi (pH 7), 20% (v/v) glycerol, 5 mM malate, 2 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 4% (w/v) PEG-8000, and 2 mM PMSF. *Buffer B:* 10 mM KPi (pH 7.5), 20% (v/v) glycerol, 5 mM malate, 2 mM DTT, 1 mM EDTA, and 10 mM $MgCl_2$. *Buffer C:* 40 mM KPi (pH 7), 1 mM EDTA, 1 mM EGTA, 30 mM NaF, 30% (saturation) $(NH_4)_2SO_4$, and 1 mM DTT. *Buffer D:* 20 mM KPi (pH 7), 1 mM EDTA, 1 mM EGTA, 30 mM NaF, 15% (v/v) glycerol, 1 mM DTT, and 20% (v/v) ethylene glycol. *Buffer E:* buffer B plus 50 mM KCl and 0.04% (w/v) NaN_3. *Buffer F:* buffer B without glycerol adjusted to pH 7.0. *Buffer G:* 100 mM Hepes-NaOH (pH 7), 5 mM $MgCl_2$, 5 mM $KHCO_3$, 5 mM DTT, and 0.04% (w/v) NaN_3. *Buffer H:* buffer G plus 64 mM $(NH_4)_2SO_4$ and 200 mM KPi. *Buffer I:* buffer H plus 5 mM malate.

Partial Purification of Nodule PEPC

All procedures were carried out at 4°C. Soybean nodules (20 g) were homogenized with an 'Osterizer' kitchen blender in 40 mL of buffer A. The homogenate was centrifuged at 24,000*g* for 20 min. The supernatant fluid, designated the crude extract, was brought to 20% (w/v) PEG with a 50% (w/v) solution of PEG-8000 containing 50 mM K-Pi (pH 7) and 2 mM EDTA. This was stirred for 20 min and then centrifuged as above. The pellet was retained and dissolved in buffer B. This solution was adsorbed, at a flow rate of 2.0 mL·min^{-1}, onto a column (1.5 × 7.5 cm) of Q-Sepharose equilibrated with buffer B. The column was connected to a FPLC system, washed with buffer B until the A_{280} decreased below 0.1 and then eluted with a 240-mL linear gradient of 0 to 400 mM KCl in buffer B. Pooled fractions (8 mL/fraction) from the Q-Sepharose column were applied, at a flow rate of 0.3 mL·min^{-1}, onto a column (1.0 × 6.0 cm) of Phenyl-Sepharose equilibrated with buffer C. The column was connected to the FPLC system and washed with a mixture of 20% buffer C and 80% buffer D until the A_{280} returned to the baseline. PEPC was then eluted with buffer D. Pooled fractions (1.8 mL/fraction) were concentrated to 0.2 mL using an Amicon ultrafiltration device fitted with a YM-30 membrane. The concentrated enzyme was applied, at 0.2 mL·min^{-1}, onto a Pharmacia prepacked Superose 6 HR 10/30 column equilibrated with buffer E. Fractions (0.4 mL) from the Superose 6 column having PEPC activity were pooled and aliquots, first frozen in liquid N_2, were stored at −80°C.

SDS-PAGE and Western Blotting

SDS-PAGE and Western blotting were performed as previously described except that a polyvinylidene difluoride membrane (Whatman) was used in place of nitrocellulose

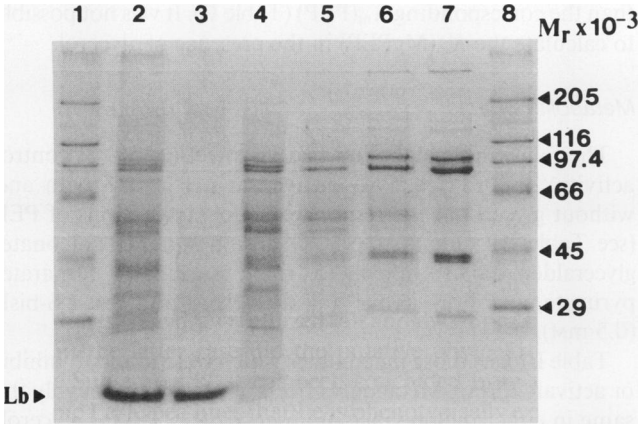

Figure 1. SDS polyacrylamide mini-gel electrophoresis of various fractions obtained during the partial purification of soybean nodule PEPC and listed in Table I. Lanes 1 and 8, molecular mass standards; lane 2, crude extract (25 μg protein); lane 3, 20% (v/v) PEG supernatant fluid (9 μg protein); lane 4, 4 to 20% (w/v) PEG pellet (22 μg protein); lane 5, Q-Sepharose pool (8 μg protein); lane 6, phenyl Sepharose pool (9 μg protein); lane 7, Superose 6 pool (12 μg protein). Proteins were stained with Coomassie blue R-250. Lb, leghaemoglobin.

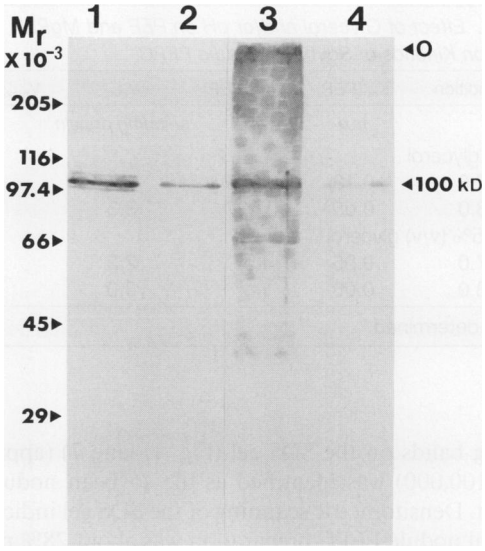

Figure 2. Western blot of soybean nodule PEPC purified through the Superose 6 step and maize leaf PEPC (Sigma Chemical Co., specific activity = 3.8 units·mg protein^{-1}). Proteins were resolved by SDS-PAGE and blot-transferred to a polyvinylidene difluoride membrane. Western analysis was performed using a 1:160 dilution of rabbit anti-(maize leaf PEPC) immune serum. Immunoreactive proteins were visualized using an alkaline phosphatase-linked secondary antibody as previously described (17). Phosphatase staining was for 2 min at 37°C. Lane 1, nodule PEPC (1.2 μg protein); lane 2, nodule PEPC (0.12 μg protein); lane 3, maize PEPC (0.7 μg protein); lane 4, maize PEPC (0.07 μg protein). O, origin.

(17). Rabbit anti-(maize leaf PEPC) immune serum was a gift from Dr. R. Chollet, University of Nebraska. Densitometric scanning of the SDS-PAGE gel of the final PEPC preparation was performed using a LKB Ultroscan XL Enhanced Laser Densitometer. Densitometric data were analysed using LKB Gelscan XL software (version 2.0).

RESULTS

Purification and Physical Properties

The maximal activity of PEPC in soybean root nodules was approximately 3.4 units·g fresh weight^{-1}. As bacteroids apparently lack PEPC (12), no special precautions were taken to prevent breakage of the bacteroids during nodule homogenization. The PEG fractionation step essentially removed all contaminating leghaemoglobin which remained in the 20% (w/v) PEG supernatant fluid (Fig. 1, lanes 3 *versus* 4). PEPC eluted as a single activity peak under all chromatographic conditions used in the purification scheme. On the Superose 6 HR 10/30 gel filtration column, the elution volume of PEPC was identical to that of ferritin (M_r = 440,000). As shown in Table I the enzyme was purified 40-fold to a specific activity of 5.2 units·mg protein^{-1} and an overall yield of 22%. The final PEPC preparation was free of contaminating aldolase, triose-P isomerase, PGA mutase, enolase, and PEP phosphatase, whilst hexose-P isomerase activity was less than 2% of PEPC activity.

SDS-PAGE and Western Blotting

Four major protein staining bands were present following SDS-PAGE of the final enzyme preparation (Fig. 1, lane 7). Western blots of the same preparation were probed with rabbit anti-(maize leaf PEPC) immune serum and revealed only one immunoreactive polypeptide, which comigrated with the 100 kD subunit of authentic maize leaf PEPC (18) (Fig. 2). Therefore, the slowest migrating of the two upper major protein

Table I. *Partial Purification of Soybean Nodule PEP Carboxylase*

Fraction[a]	Volume	Activity[b]	Protein	Specific Activity	Purification	Yield
	mL	*units*	*mg*	*units/mg*	*-fold*	%
Crude extract	42	67	531	0.13	1	100
4–20% PEG fraction	35	65	253	0.26	2	97
Q-Sepharose	76	50	59	0.85	6.5	75
Phenyl sepharose	14	21	8.3	2.6	20	31
Superose 6	1.2	15	2.8	5.2	40	22

[a] See Figure 1. [b] Enzyme activity was measured using the standard PEPC assay described in "Materials and Methods."

Table II. *Effect of Glycerol and/or pH on PEP and MgPEP Saturation Kinetics of Soybean Nodule PEPC*

Condition	K_m (PEP)	K_m (MgPEP)	V_{max}	V_{max}/K_m (PEP)
	mM	mM	units/mg protein	
Minus glycerol				
pH 7.0	0.13	0.069	1.7	13
pH 8.0	0.05	0.027	3.3	66
Plus 15% (v/v) glycerol				
pH 7.0	0.05	ND[a]	2.2	44
pH 8.0	0.06	ND[a]	3.0	50

[a] Not determined.

staining bands on the SDS gel (Fig. 1, lane 7) (approximate M_r = 100,000) was identified as the soybean nodule PEPC subunit. Densitometric scanning of the SDS gel indicated that the final nodule PEPC preparation was about 28% pure.

Attempts to Demonstrate Different Oligomeric Forms of Soybean Nodule PEPC

PEPC purified through the Superose 6 step was rechromatographed on the same column in the presence or absence of 20% (v/v) glycerol, high ionic strength, or malate in an attempt to demonstrate different oligomeric forms of the enzyme. The protein concentration loaded onto the column was decreased to 0.23 mg·mL^{-1} and the column was separately equilibrated with buffers F to I listed under "Materials and Methods." Under no circumstance was the elution volume of PEPC altered.

Kinetic Properties

Effect of pH

PEPC activity assayed in the presence of saturating (2.5 mM) PEP in the standard Bis-Tris-Propane assay buffer (see "Materials and Methods") with and without 15% (v/v) glycerol had a broad pH optimum of between pH 8.0 and 9.5. At pH 7, in either the presence or absence of glycerol, approximately 70% of maximum activity remained. Activity was negligible at pH 6.

PEP Saturation Kinetics

PEP saturation kinetics were analyzed using the Hepes assay buffer (see "Materials and Methods"). Typical Michaelis-Menten kinetics were observed under all four assay conditions, *i.e.* pH 7 or 8 with and without 15% (v/v) glycerol (data not shown). Table II lists K_m(PEP), V_{max}, and V_{max}/K_m values for the four different assay conditions. The highest K_m(PEP) and lowest V_{max} were obtained at pH 7 in the absence of glycerol. Either raising the pH to 8 or adding glycerol at pH 7 halved the K_m(PEP) and markedly increased V_{max}/K_m. Glycerol and pH 8 together had no greater effect on the K_m(PEP) than either of these factors alone. Glycerol did not markedly alter the V_{max} at either pH 7 or pH 8. Raising the pH to 8 almost doubled V_{max}. In the absence of glycerol at pH 7 or pH 8 the K_m(MgPEP) was found to be about two-fold lower than the corresponding K_m(PEP) (Table II). It was not possible to calculate the K_m(MgPEP) in the presence of glycerol.

Metabolite Effects

The following metabolites had no effect (±15% of control activity) on PEPC activity assayed at pH 7 or 8 with and without glycerol at the respective K_m concentrations of PEP (see Table II): Gln, Asn, Ser, Gly, Arg, Ala, 6-P-gluconate, glyceraldehyde-3-P (all at 5 mM); succinate, fumarate, pyruvate, and Fru-1,6-bisP (all at 4 mM); and Fru-2,6-bisP (0.5 mM).

Table III lists those metabolites which were found to inhibit or activate PEPC. Metabolite effects at pH 8 were roughly the same in either the presence or absence of 15% (v/v) glycerol. The enzyme was generally more responsive to inhibitors or activators at pH 7 in the absence of glycerol than under the other assay conditions. The exceptions were 3-PGA, 2-PGA, and Pi which were more inhibitory if the pH was raised to 8 or if glycerol was added at pH 7. Pi was an activator at pH 7 in the absence of glycerol. Raising the pH to 8 or adding glycerol completely abolished the effects of the activators and decreased or abolished the effects of the inhibitors (except for 3-PGA, 2-PGA, and Pi).

Activators

At pH 7 in the absence of glycerol, Glc-6-P and Fru-6-P each almost doubled PEPC activity (Table III), but the K_a of Glc-6-P was 6.5-fold greater than that of Fru-6-P (Table IV). Glc-1-P and DHAP each caused an approximately 50% increase in PEPC activity (Table III). The K_a of Glc-1-P was an order of magnitude less than the K_a of DHAP which was similar to that of Fru-6-P (Table IV). None of these metabo-

Table III. *Effect of Various Metabolites on Activity of Soybean Nodule PEPC*

Assays were conducted at pH 7 or 8 in the presence and absence of 15% (v/v) glycerol at the respective K_m concentrations of PEP (see Table II). Enzyme activity is expressed relative to the respective control set at 100.

Metabolite	Concentration	Relative activity			
		−glycerol	+glycerol	−glycerol	+glycerol
	mM	pH 7.0		pH 8.0	
Glc-6-P	4	189	93	103	105
Fru-6-P	4	187	106	105	128
Glc-1-P	4	154	102	106	107
DHAP	4	162	105	102	107
3-PGA	5	97	41	56	42
2-PGA	5	118	60	54	52
Malate	1.25	4	39	102	92
Asp	1.25	40	83	92	105
Glu	1.25	60	87	106	103
Citrate	2.5	29	47	65	57
2-OG	5	23	55	86	82
Pi	10	132	81	74	76
	20	152	63	57	54
	30	139	62	36	38
	40	120	44	56	51

Table IV. *Kinetic Constants for Several Effectors of Soybean Nodule PEPC*

Assays were conducted at pH 7 in the presence and absence of 15% (v/v) glycerol or at pH 8 in the absence of glycerol and at the respective K_m concentrations of PEP (see Table II).

Effector	−glycerol pH 7.0		−glycerol pH 8.0		+glycerol pH 7.0	
	I_{50}	K_a	I_{50}	K_a	I_{50}	K_a
			mM			
Glc-6-P		1.3		NE[a]		NE
Fru-6-P		0.2		NE		NE
Glc-1-P		0.04		NE		NE
DHAP		0.4		NE		NE
3-PGA	NE		6.4		3.4	
Malate	0.2		4.8		0.7	
Asp	0.9		NE		3.2	
Glu	1.6		NE		5.0	
Citrate	1.1		3.1		1.5	
2-OG	2.2		NE		5.7	

[a] No effect.

lites had any effect on PEPC activity at pH 8 or at pH 7 in the presence of 15% (v/v) glycerol (Table III).

Pi also activated PEPC at pH 7 in the absence of glycerol with maximal activation occurring at 20 mM Pi (Table III). At higher concentrations, the extent of Pi activation declined. This decline in Pi activation could be due to chelation of Mg^{2+} by Pi since it has been shown that Mg-PEP is the preferred substrate for maize leaf PEPC (28). However, the calculated concentrations of Mg^{2+} remaining in the assay after chelation by Pi at pH 7 in the absence of glycerol were 0.90, 0.39, 0.24, and 0.18 mM at 10, 20, 30, and 40 mM Pi, respectively. The corresponding values at pH 8 in the absence of glycerol were 1.09, 0.49, 0.31, and 0.23 mM, respectively. It was not possible to calculate these values in the presence of glycerol. Therefore, given that the concentration of PEP in the assay was 0.13 and 0.05 mM at pH 7 and 8, respectively, it is unlikely that the effects of high concentrations of Pi are due to chelation of Mg^{2+}.

Inhibitors

There were three classes of inhibitors. The first class, which included malate, Asp, Glu, and 2-OG, had low I_{50} values at pH 7 in the absence of glycerol (Table IV). Adding 15% (v/v) glycerol at pH 7 increased their I_{50} values 2.6- to 3.6-fold and adjusting the pH to 8 in the absence of glycerol abolished the inhibition or at least caused an order of magnitude increase in the I_{50}. The second class of inhibitors contained only citrate which gave similar I_{50} values under all assay conditions (Table IV). The third class included 3-PGA, 2-PGA, and Pi which were inhibitory under all assay conditions except for pH 7 in the absence of glycerol (Table III). Similar I_{50} values were obtained for 3-PGA under both inhibitory assay conditions (Table IV).

DISCUSSION

Soybean nodule PEPC eluted as a single activity peak under all chromatographic conditions used in the present study. This is consistent with previous reports for soybean and alfalfa nodule PEPC (16, 25), but contrasts with results obtained with French bean and lupin nodules where two isoforms of PEPC were resolved by ion exchange chromatography (6, 11). Our final preparation was judged to be approximately 28% pure, and had a specific activity of about 5 units·mg protein^{-1}. This value is about eightfold lower than that previously obtained for PEPC purified from soybean and alfalfa nodules (16, 25).

The native molecular mass of soybean nodule PEPC was estimated to be 440 kD. Western blotting experiments indicated a subunit molecular mass of about 100 kD for the soybean nodule enzyme (Fig. 2), confirming the results of Miller *et al.* (14). Thus, soybean nodule PEPC appears to be a homotetramer of 100 kD subunits.

As has been demonstrated for maize and wheat leaf PEPC (28), it is probable that the MgPEP complex is the preferred form of the substrate for the soybean nodule enzyme. The K_m(MgPEP) for the soybean enzyme was about twofold lower than the K_m(PEP) at either pH 7 or pH 8 (Table II). Raising the pH to 8 and/or adding 15% (v/v) glycerol to the assay approximately halved the K_m(PEP) to about 50 μM (Table II). The effects of pH 8 and glycerol were not additive, suggesting that they act by the same mechanism. Previously reported K_m(PEP) values for soybean and alfalfa nodule PEPC, assayed at pH 7.5 in the absence of glycerol, were 0.094 and 0.21 mM, respectively (16, 25). PEPC I and PEPC II from lupin nodules had K_m(PEP) values of 0.09 and 0.18 mM, respectively, at pH 8 in the absence of glycerol (11).

Similar to soybean nodule PEPC (Tables II–IV), the K_m(PEP) and sensitivity to most metabolite effectors of the maize leaf enzyme show a significant reduction from pH 7 to pH 8 or when glycerol is added to the assay medium at pH 7 (18). In maize this has been attributed to displacement of the equilibrium between a dimeric (200 kD) and a tetrameric (400 kD) form of PEPC toward the tetramer (18). We were unable to detect lower molecular mass forms of soybean nodule PEPC when the 440-kD enzyme was rechromatographed on the Superose 6 gel filtration column at pH 7 in the absence of glycerol. However, protein concentration also influences the aggregation state of maize leaf PEPC (13) and this could explain why no lower molecular mass forms of soybean nodule PEPC in our partially purified preparation were detected.

The concentration of several metabolites in the plant fraction of soybean nodules was calculated using the data of Streeter (23), and assuming a fresh weight:dry weight ratio of 5:1 and a volume to fresh weight conversion of 1.2 g·cm^{-3} (Table V). These values were compared with the K_a and I_{50} values obtained for several effectors of partially purified soybean nodule PEPC (Table IV) to obtain an indication of the possible *in vivo* significance of the effectors. The calculated concentrations do not take into account subcellular compartmentation which may have a significant effect on local concentration. The I_{50}(malate) at pH 7 in the absence of glycerol was about 10-fold lower than its *in vivo* concentration suggesting that malate is likely to be an important regulator of PEPC *in vivo*. At pH 8, however, the I_{50}(malate) was about 50% greater than its *in vivo* concentration. These data indicate that pH fluctuations may be significant for the regulation of

Table V. *Metabolite Concentrations in the Plant Fraction of Soybean Nodules*

Metabolite	Concentration[a]
	mM
Malate	3.35
Citrate	0.62
Fumarate	0.66
Succinate	0.42
Aspartate	3.24
Glutamate	1.35

[a] Calculated from the data of Streeter (23).

PEPC by malate. This is consistent with the proposed pH stat function of soybean nodule PEPC (8). Similar comparisons between the data of Tables IV and V suggest that Asp and Glu, but not citrate, could be important inhibitors of PEPC *in vivo,* especially at pH 7.

Our findings are in contrast to those of Peterson and Evans (16) who found no effectors for soybean nodule PEPC. One explanation for this may be that saturating concentrations of PEP were used in the earlier study. Like us, Marczewski (11) found that malate and Asp inhibition of lupin nodule PEPC was pH dependent. Pyruvate, 2-OG, and Glu also inhibited lupin nodule PEPC, but their effects were not pH dependent.

Metabolite Regulation of PEPC in Relation to Nodule Metabolism

Figure 3 presents a model summarizing the regulatory mechanisms of soybean nodule PEPC which may be most important in controlling anaplerotic carbon flow in support N_2 fixation. The regulatory design of this enzyme appears to be well suited to its central role in supplying the bacteroids with respiratory substrates, as well as for providing carbon skeletons for NH^+-assimilation via GS/GOGAT. N_2 fixation is dependent on currently supplied photosynthate in the form of sucrose rather than stored starch reserves (19). Therefore, hexose-P activation of soybean nodule PEPC could coordinate sucrose availability with the rate of synthesis of dicarboxylic acids required by the bacteroids to support nitrogenase activity. Triose-P activation may also contribute to this feed-forward regulation of soybean nodule PEPC.

In contrast to the other effectors, 3-PGA and 2-PGA inhibited PEPC at pH 8 or at pH 7 in the presence of glycerol, but had no effect at pH 7 in the absence of glycerol (Tables III and IV). Ureide biosynthesis from the oxidative catabolism of purines requires 3-PGA as an input at the level of plastidic 3-PGA dehydrogenase (21). Therefore, if ureide biosynthesis is curtailed for some reason, such as decreased NH_4^+ supply to the plant by the bacteroids, then 3-PGA might accumulate in the plastid. This accumulation in the plastid could be transmitted to the cytosol via a triose-P/Pi translocator, which may lead to PEPC inhibition and consequent decreased synthesis of dicarboxylic acids. In this way respiratory substrate supply to the bacteroids by the plant would be coordinated with NH_4^+ supply to the plant by the bacteroids.

Similarly, malate inhibition of nodule PEPC may provide a tight feedback control which closely balances PEPC activity with the rate of utilization of respiratory substrates by the bacteroids. Asp inhibition is easy to understand in amide transporting symbioses because of the direct involvement of nodule PEPC in Asn synthesis (3). However, in soybeans, which transport ureides (21), the significance of Asp inhibition of PEPC is not immediately apparent. Asp accumulation may simply reflect decreased oxaloacetate utilization via malate dehydrogenase or citrate synthase.

Inhibition by Glu and 2-OG is consistent with a function for soybean nodule PEPC to replenish TCA cycle intermediates withdrawn in the form of 2-OG to support NH_4^+-assimilation via GS/GOGAT. When NH_4^+ is available, Glu should be rapidly converted to Gln via GS. If 2-OG availability limits GOGAT activity then Gln levels may rise and Glu levels fall. These low Glu and 2-OG levels could serve to activate soybean nodule PEPC, thus replenishing the TCA cycle and alleviating the limitation on GOGAT activity imposed by 2-OG availability. A rationale similar to this was used to explain the regulation of *Selenastrum minutum* (a green alga) PEPC activity during NH_4^+-assimilation (22, 26). The algal PEPC was activated by Gln, but was inhibited by Glu and 2-OG. We proposed that a high Gln/Glu ratio signaled 2-OG limitation of GOGAT activity and the need for increased anaplerotic activity of PEPC (22, 26). During NH_4^+-assimilation by *S. minutum* Gln accumulates to high levels (24, 26), but it is unlikely that this would occur in legume nodules. In amide-transporting symbioses Gln would be exported in the xylem, whereas in ureide-transporting symbioses it would be incorporated into the purine precursor of ureides (21). Accumula-

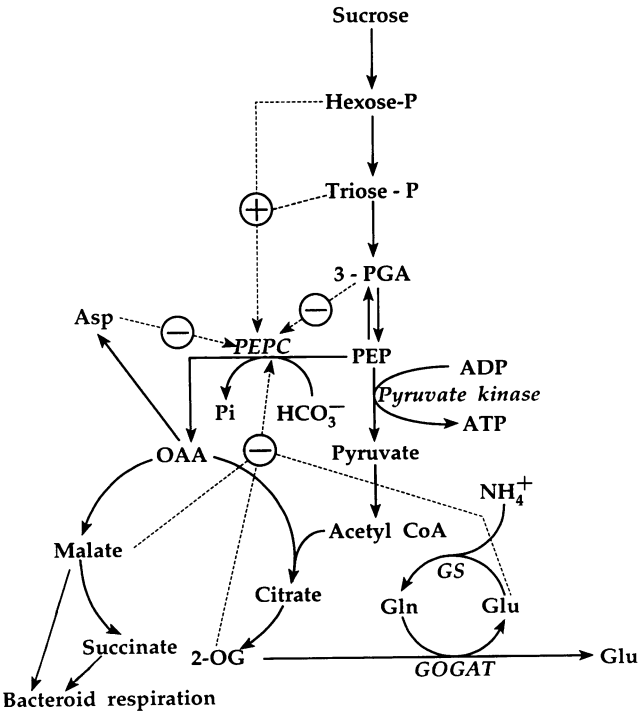

Figure 3. A model for the *in vivo* metabolite regulation of soybean nodule PEPC. Solid lines represent the flow of metabolites whereas dashed lines indicate regulation of PEPC by effectors; (⊕), activation, (⊖), inhibition.

tion of Gln in nodules due to decreased ureide export in the xylem or decreased ureide biosynthesis would not necessarily coincide with a need for elevated PEPC activity. This may be why Gln (at 5 mM) does not affect soybean nodule PEPC *in vitro*.

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