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PHOSPHO*ENOL*PYRUVATE CARBOXYLASE FROM SOYBEAN NODULE CYTOSOL

EVIDENCE FOR ISOENZYMES AND KINETICS OF THE MOST ACTIVE COMPONENT

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Summary

Phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) from plant cells of soybean nodules was studied to assess its role in providing carbon skeletons for aspartate and asparagine synthesis. The enzyme was purified 119-fold by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose, BioGel A-1.5m, and hydroxyapatite chromatography. Five activity bands were resolved with discontinuous polyacrylamide gel electrophoresis. A small quantity of enzyme from the most active band was separated from the others by preparative electrophoresis. The apparent Michaelis constants of this enzyme for phospho*enol*pyruvate and HCO_3^- were $9.4 \cdot 10^{-2}$ and $4.1 \cdot 10^{-1}$ mM, respectively. A series of metabolites tested at 1 mM had no significant effect on enzyme activity. These experiments indicate that the major factors directly controlling phospho*enol*pyruvate carboxylase activity in vivo are phospho*enol*pyruvate and HCO_3^- concentrations.

Introduction

Recent experimentation has shown that cytosol phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) may play an essential role in the metabolism of legume root

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nodules during fixation of atmospheric N_2 [1,2]. Its role appears to be that of diverting phospho*enol*pyruvate from the terminal steps of glycolysis to the synthesis of oxaloacetic acid. Oxaloacetic acid would then serve as a carbon skeleton for aspartate and asparagine synthesis. To define factors affecting phospho*enol*pyruvate utilization and direction of carbon flow, an attempt was made to purify and study the properties of soybean nodule cytosol phospho*enol*pyruvate carboxylase.

Materials and Methods

Plant culture. Soybeans (*Glycine max* L. var. Chippewa) were grown as described previously [3]. Plants were inoculated with peat cultures of *Rhizobium japonicum* (USDA 110).

Reagents. Imidazole (Sigma, St. Louis, MO) was recrystallized from ethyl acetate. Bovine serum albumin was also obtained from Sigma. Polyvinylpolypyrollidone was purchased from GAF Corp. (New York) and washed with acid [4].

Electrophoresis. Polyacrylamide gels were used for separation of multiple forms of phospho*enol*pyruvate carboxylase. The following buffer systems were employed: Running gel (0.6 × 10 cm): 176 mM imidazole/HCl, 10% (v/v) glycerol (pH 7.3). Stacking gel (0.5 cm): 176 mM imidazole/HCl, 10% (v/v) glycerol (pH 6.1). Lower (anode) reservoir buffer was 25 mM imidazole/phosphate (pH 7.3). Upper (cathode) reservoir buffer was 5 mM imidazole/glycylglycine (pH 7.1). When protein entered the running gel, either phospho*enol*pyruvate (0.1 or 0.2 mM) was added to the upper reservoir buffer, or the buffer was changed to 20 mM potassium phosphate (pH 7.1). Both procedures served to stabilize the phospho*enol*pyruvate carboxylase activity. Enzyme activity bands were located by incubating each gel with 10 ml phospho*enol*pyruvate carboxylase reaction mixture (without NADH and malate dehydrogenase, EC 1.1.1.37) and 0.5 ml 65 mM Fast Violet B salt [5].

Purification of phosphoenolpyruvate carboxylase. Purification steps were similar to others [6—9] for plant phospho*enol*pyruvate carboxylases. All procedures through the $(NH_4)_2SO_4$ precipitation steps were conducted at 0—4°C. Except where indicated, the buffer was potassium phosphate (pH 7.2), 10% (v/v) glycerol.

Nodules were harvested from 32—35 day-old plants, rinsed thoroughly with distilled water, and ground (in 40-g batches) with 1/3 amount (w/v) polyvinylpolypyrollidone and 3 vols. 100 mM buffer (25% v/v glycerol, 5 μM dithiothreitol), for 30 s in an Omni-mixer. The resulting slurry was filtered through cheesecloth and centrifuged at 37 000 × *g* for 10 min. The supernatant was removed and precipitates forming at 40, 45, and 55% saturation $(NH_4)_2SO_4$ were prepared. The 55% pellet was suspended in 200 mM buffer, frozen in liquid N_2, and stored at —79°C. All subsequent steps were performed at room temperature. After thawing, the buffer was changed to 20 mM on a BioGel P-6 column and the enzyme applied to a 2.1 × 5.7 cm DEAE-cellulose column (equilibrated with 20 mM buffer). A stepwise gradient of 90 ml vols. of 20, 60, 150, 200, and 300 mM buffer was used to elute the activity from the column. The 150 mM fraction was precipitated with 60% $(NH_4)_2SO_4$.The resuspended pellet

was chromatographed on a 2.2 × 65 cm BioGel A-1.5m column, developed with 20 mM buffer. Fractions containing at least halfmaximal (peak) activity were pooled and applied to a 1.5 × 2.9 cm hydroxyapatite column (equilibrated with 20 mM buffer, glycerol omitted). A 150 ml linear gradient of 20—400 mM buffer (glycerol omitted) seperated phospho*enol*pyruvate carboxylase activity from the major 280 nm absorbing peak. Peak fractions were pooled, made to 10% (v/v) glycerol, frozen in liquid N_2, and stored at —79°C. Electrophoresis showed the presence of five separate activity bands.

The multiple enzyme forms were not resolved by gel filtration or gradient-eluted ion-exchange columns. Therefore, a preparative electrophoresis technique was employed. Approx. 48 units frozen enzyme were contracted (PM-30 Amicon filter) and electrophoresed in nine tubes (approx. 80 μg protein/tube) as described above, with 0.2 mM phospho*enol*pyruvate added to the upper reservoir buffer. The desired enzyme form was removed from the gel sections by electrophoresing into tied sections of dialysis tubing, which had been placed over the anode end of the glass tubes. Each dialysis tube segment contained 1 ml 200 mM imidazole/phosphate (pH 7.2). After electrophoresis, the pooled enzyme solutions (three units total) were made to 10% glycerol, frozen in liquid N_2, and stored at —79° C.

Enzyme assays. A colorimetric and a coupled enzyme assay were both used for measurement of phospho*enol*pyruvate carboxylase activity. The 2,4-dinitrophenylhydrazine (colorimetric) assay was similar to that described by Kachmar and Boyer [10] for the detection of pyruvate. The enzyme reaction was in 1 ml and was terminated with addition of 1 ml 0.0125% 2,4-dinitrophenylhydrazine in 1 M HCl. After 10 min at 30° C, 0.4 ml 3.5 M NaOH was added. The absorbance was read at 414 nm after 10 min at 30° C. The standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM $KHCO_3$, and 2 mM cyclohexylamine phospho*enol*pyruvate. Coupled enzyme assays contained the same concentrations of reagents with 0.16 mM NADH and ten units pig heart malate dehydrogenase (Sigma) in a final volume of 2 ml. Activity was measured as the loss of absorbance at 340 nm. β-Hydroxy butyrate dehydrogenase (EC 1.1.1.30) was measured by the method of Wong and Evans [11]. One unit of enzyme activity is defined as that forming 1 μmol product/ min at 30° C.

Protein measurement. Protein was determined by the method of Lowry et al. [12]. For measurements from crude extracts, the protein was first precipitated with trichloroacetic acid.

Results

Multiple enzyme forms

Enzyme purified through the hydroxyapatite step (Table I) was electrophoresed and the gels stained for enzyme activity and protein (Fig. 1). The enzymically active regions of the gels were subjected to electrophoresis in a second direction (Fig. 1). With the exception of band IIIb, they ran as distinct enzymes. Band IIIb, when electrophoresed in the second direction, gave a wide band of activity in regions corresponding to band IIIa and IIIb. Although it is likely that this was due to a lack of resolution in the first direction, it can only

TABLE I

PARTIAL PURIFICATION OF CYTOSOL PHOSPHO*enol*PYRUVATE CARBOXYLASE ACTIVITY FROM ROOT NODULES

96 g nodules were harvested from 33 day-old soybean plants.

Purification step	Total units	Total protein (mg)	Specific activity (unit/mg)	Recovery (%)
Crude enzyme	620	1728	0.36	
$(NH_4)_2SO_4$, 45—55%	417	322	1.30	67
DEAE-cellulose, 150 mM potassium phosphate	213	61	3.49	34
BioGel A-1.5 m	103	8.6	12.0	17
Hycroxyapatite	64	1.5 *	42.7	10

* Estimated form the A_{280} profile of column eluate and the total protein applied to the column.

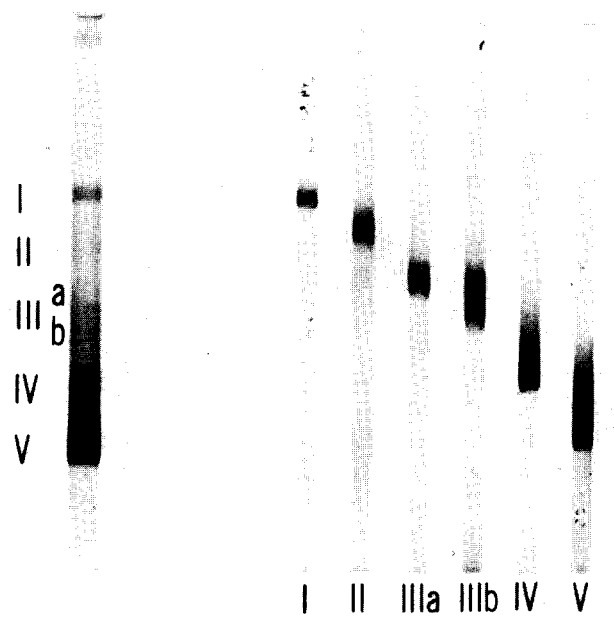

Fig. 1. Activity-stained polyacrylamide gels after one- and two-dimensional electrophoresis of partially purified soybean nodule cytosol phospho*enol*pyruvate carboxylase activity. Enzyme purified through the hydroxyapatite step (Table I) was electrophoresed on 5% polyacrylamide gels with 0.2 mM phospho-*enol*pyruvate added to the upper (cathode) reservoir buffer. Approx. 0.12 unit (40 μg protein) was applied to each gel. One first-dimension gel (far left) was stained for activity. For electrophoresis in the second direction, 4-mm gel sections were cut from an unstained gel. They were than placed on 12-cm gels of the same concentration polyacrylamide and containing the same buffer. Electrophoresis was then continued as in the first dimension. Activity stain was added with enzyme reaction mixture to the first direction gel, and 20 min after the reaction mixture for second direction gels. In each case the electrophoretic 'front' is marked by a darkened area that had the same mobility as bromophenol blue. Gels are arranged (inverted) and bands numbered according to I.U.B. rules for isoenzyme nomenclature. I—V represent the anodic to cathodic bands, respectively. A gel analogous to the first dimension gel above was stained for protein with Coomassie blue. Dark bands appeared at the origin of the running gel and at the phospho-*enol*pyruvate carboxylase V location. There was one light band between these two and one near the 'front'. Light, broad bands were visible at phospho*enol*pyruvate carboxylase IV and IIIa regions of the gel.

be concluded that five of the six bands in Fig. 1 were not generated from other enzyme forms during electrophoresis. Furthermore, they do not seem to represent a dynamic equilibrium between several forms (e.g. subunit combinations) of phospho*enol*pyruvate carboxylase. Individual enzyme sections cut from one-dimensional gels gave phospho*enol*pyruvate carboxylase activity when incubated with the coupled enzyme reaction mixture. The enzyme preparation contained no lactate dehydrogenase (EC 1.1.1.27 and 1.1.1.28), malate dehydrogenase, pyruvate kinase (EC 2.7.1.40), or phospho*enol*pyruvate phosphatase activities. Therefore, the bands appeared to be due to phospho*enol*-pyruvate carboxylase activities.

The gels in Fig. 1 were run with phospho*enol*pyruvate added to the upper reservoir buffer. When phosphate was used in the alternative procedure for enzyme stabilization, the one-dimensional pattern on 3.5% gels was almost identical to that in Fig. 1 (5% gels). The procedure using phosphate gave noticeably greater stabilization but the use of phospho*enol*pyruvate gave better resolution. When potassium phosphate was used in the same manner and at the same concentration (0.2 mM) as phospho*enol*pyruvate, activity on the gels was barely detectable.

Estimates of relative amounts of the multiple forms were made in an attempt to evaluate the relative significance of each to cellular metabolism. The most cathodic enzyme (band V) accounted for approx. 50% of total carboxylase activity in the gels (Table II). This activity band also corresponded to a main band in gels stained for protein (Fig. 1).

The possibility exists that one or more of the detected multiple forms arose from breakage of bacteriods or mitochondria during the isolation procedure. Activities of β-hydroxybutyrate dehydrogenase (bacteriod enzyme) and phospho*enol*pyruvate carboxylase were compared in crude cytosol and soni-

TABLE II

RELATIVE AMOUNTS OF SOYBEAN NODULE CYTOSOL PHOSPHO*enol*PYRUVATE CARBOXYLASE MULTIPLE FORMS AFTER ELECTROPHORETIC SEPARATION

Activity (1.2 units, 136 μg protein/gel) from an experiment involving anaerobic isolation and precipitation was electrophoresed with 0.2 mM phospho*enol*pyruvate added to the upper reservoir buffer. The enzyme preparation was a derivation of a standard procedure involving isolation, $(NH_4)_2SO_4$ precipitation and BioGel A-1.5m chromatography as described for purification. One gel was stained for activity and 4-mm sections corresponding to individual bands were cut from two unstained gels. The sections were each gently shaken at room temperature with 4 ml enzyme reaction mixture (colorimetric assay) for 1 h. A section of the gel between enzyme I and the electrophoretic 'front' was used as control. Control values were substracted from individual enzyme values.

Phospho*enol*-pyruvate carboxylase	Oxaloacetic acid (μmol synthesized/gel slice)		Relative amount as percent of total
	Gel No. 1	Gel No. 2	
I	0.124	0.072	9
II	0.080	0.080	7
IIIa	0.060	0.064	6
IIIb	0.124	0.126	12
IV	0.160	0.172	15
V	0.504	0.580	50

cated bacteroid extracts. Calculations from these data showed that bacteriod phospho*enol*pyruvate carboxylase probably did not contribute more than 0.2% to the total activity in cytosol extracts. The data in Table II show that no enzyme form contributed less than 6% of the total activity. Therefore, all the detected multiple forms were very likely to be of cytosol origin. In agreement with the results of Christeller et al. [2], phospho*enol*pyruvate carboxylase activity associated with the bacteroids was barely detectable. Mitochondria, which could have been a source of activity [13,14], were not examined.

Screening for Artifacts

Protein purified through the hydroxyapatite step had an A_{280}/A_{260} ratio of 1.49, which was not as good as commercial bovine serum albumin (A_{280}/A_{260} = 1.67). This result indicated a possibility of phenol and/or quinone contamination [4,15]. It is possible that the multiple forms were artifacts caused by discrete attachment of phenols or quinones which may have been present in the crude enzyme preparation. This possibility and several others were examined by electrophoresing enzyme samples extracted and partially purified under different conditions. The basic procedure for partial purification was as described in Table II. A number of changes in the standard procedure were used which were designed to alter the possible effects of phenols or quinones [4], metal ion interactions, and low temperature. In all cases, the activity-stained gels (one dimension) were very similar to that in Fig. 1. The data strongly suggest that the multiple enzyme pattern was not artifactual.

Properties of phosphoenolpyruvate carboxylase V

Thawed phospho*enol*pyruvate carboxylase V was very unstable, losing up to 80% of its activity in 30 min at room temperature. A variety of treatments failed to significantly stabilize the activity. However, by using the colorimetric assay and rapidly initiating assays, kinetic data were obtained.

The apparent Michaelis constants of the enzyme were $9.4 \cdot 10^{-2}$ and $4.1 \cdot 10^{-1}$ mM for phospho*enol*pyruvate and HCO_3^-, respectively. These values were determined from least-squares regression analyses of Hanes plots of the data. The apparent K_m (phospho*enol*pyruvate) was similar to ($\pm 5 \cdot 10^{-2}$ mM) values reported for a number of other plant phospho*enol*pyruvate carboxylases [7—9, 16—19]. The apparent K_m (HCO_3^-) was similar to the value ($3.1 \cdot 10^{-1}$ mM) reported for peanut cotyledon enzyme [6]. Most such constants for HCO_3^- have been in the range $5 \cdot 10^{-2}$—1.5 mM [6,8,9,17] for plant enzyme. In addition, a number of metabolites were tested for their effects on enzyme activity. Concentrations of phospho*enol*pyruvate and $KHCO_3$ were lowered to 1.0 and 4.0 mM, respectively, for these tests. At 1 mM, D-fructose-6-*P*, D-fructose-1,6-P_2, ATP, ADP, L-aspartate, L-asparagine, L-glutamate, L-glutamine, citrate, isocitrate, succinate, and malate did not alter the amount of product synthesized by more than 9% when compared with controls. Similar results were obtained with 5 mM NH_4Cl. 1 mM AMP caused 12% inhibition of the activity. Pyruvate and α-ketoglutarate (both at 1 mM) were tested with the coupled enzyme assay and neither influenced activity by more than 9%.

Discussion

The evidence presented indicates that at least five distinct phospho*enol*pyruvate carboxylase enzyme forms are present in the plant cell cytoplasm of soybean root nodules. Lack of resolution on gel filtration columns suggests that the enzymes do not differ greatly in molecular weight. This fact, combined with the regular nature of the electrophoretic pattern (Fig. 1), indicate that the enzymes may be similar proteins that differ in charge properties. Such charge differences could be a result of covalent attachment of quinones or other plant products [4]. This possibility was not completely eliminated in the experiments. In addition, deamidation of glutamine and asparagine occur spontaneously in proteins [20]. Such deamidation of or quinone attachment to phospho*enol*pyruvate carboxylase V would be consistent with the observed electrophoretic pattern. Answers to these questions await the isolation and characterization of individual enzyme forms.

The kinetic properties of phospho*enol*pyruvate carboxylase V were examined on the assumption that this enzyme plays a major role in the scheme proposed earlier [21] for the utilization of phospho*enol*pyruvate in the soybean nodule cytosol. The apparent K_m for HCO_3^- was approximately 100-fold higher than that calculated for lupin nodule phospho*enol*pyruvate carboxylase [2]. With the soybean enzyme, rates without added HCO_3^- in crude and purified preparations were usually about five-fold lower than V rates. From these results and the considerations of Christeller et al. [2], it is likely that the in vivo rate of phospho*enol*pyruvate carboxylase activity in soybean nodule cytosol is limited by HCO_3^-. This property indicates that higher proportions of carbohydrate could flow to aspartate and asparagine synthesis when nodule respiration and nitrogen fixation are high.

The metabolites tested appeared to have no great effect on phospho*enol*pyruvate carboxylase V activity. However, as pointed out by Wong and Davies [19], it is possible that the purification could have affect the allosteric properties of the enzyme as also suggested by the loss of stability after purification. In addition, several of the amino acids (aspartate, asparagine, glutamine) were tested at a concentration (1 mM), much lower than those probably found in the nodules. A slight inhibition by AMP was detected and this metabolite may therefore serve as a weak feedback inhibitor, as it is generated in the asparagine synthetase reaction. However, its role as an inhibitor might be questioned because of possible equilibration with ATP and ADP via adenylate kinase. Therefore, a phospho*enol*pyruvate- and HCO_3^--limited and slightly AMP-inhibited phospho*enol*pyruvate carboxylase is tentatively added to the scheme proposed for control of pyruvate kinase and phospho*enol*pyruvate utilization [21] in soybean nodule cytosol.

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References

1 Lawrie, A.C. and Wheeler, C.T. (1975) New Phytol. 74, 437—445
2 Christeller, J.T., Laing, W.A. and Sutton, W.D. (1977) Plant Physiol. 60, 47—50
3 Evans, H.J., Koch, J.B. and Klucas, R. (1972) Methods Enzymol. 24, 470—476
4 Loomis, W.D. (1974) Methods Enzymol. 31, 528—534
5 Hatch, M.D., Osmond, C.B., Troughton, J.H. and Bjorkman, O. (1972) Carnegie Inst. Wash. Yearb. 71, 135—141
6 Maruyama, H., Easterday, R.L., Chang, H.-C. and Lane, M.D. (1966) J. Biol. Chem. 241, 2405—2412
7 Smith, T.S. (1968) Arch. Biochem. Biophys. 125, 178—188
8 Miziorko, H.M., Nowak, T. and Mildvan, A.S. (1974) Arch. Biochem. Biophys. 163, 378—389
9 Mukerji, S.K. (1977) Arch. Biochem. Biophys. 182, 343—351
10 Kachmar, J.R. and Boyer, P.D. (1953) J. Biol. Chem. 200, 669—682
11 Wong, P.J. and Evans, H.J. (1971) Plant Physiol. 47, 750—755
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Mazelis, M. and Vennesland, B. (1957) Plant Physiol. 32, 591—600
14 Brandon, P.C. (1967) Plant Physiol. 42, 977—984
15 Pierpont, W.S. (1969) Biochem. J. 112, 619—629
16 Ting, I.P. (1968) Plant Physiol. 43, 1919—1924
17 Mukerji, S.K. and Ting, I.P. (1971) Arch. Biochem. Biophys. 143, 297—319
18 Ting, I.P. and Osmond, C.B. (1973) Plant Physiol. 51, 448—453
19 Wong, K.F. and Davies, D.D. (1973) Biochem. J. 131, 451—458
20 Midelfort, C.F. and Mehler, A.H. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1816—1818
21 Peterson, J.B. and Evans, H.J. (1978) Plant Physiol. 61, 909—914 (62, 320)