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Short Communication

Alfalfa Root Nodule Carbon Dioxide Fixation[1]

II. PARTIAL PURIFICATION AND CHARACTERIZATION OF ROOT NODULE PHOSPHOENOLPYRUVATE CARBOXYLASE

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ABSTRACT

A nonphotosynthetic phosphoenolpyruvate carboxylase (EC 4.1.1.31) was partially purified from the cytosol of root nodules of alfalfa. The enzyme was purified 86-fold by ammonium sulfate fractionation, DEAE-cellulose, hydroxylapatite chromatography, and reactive agarose with a final yield of 32%. The enzyme exhibited a pH optimum of 7.5 with apparent K_m values for phosphoenolpyruvate and magnesium of 210 and 100 micromolar, respectively. Two isozymes were resolved by nondenaturing polyacrylamide disc gel electrophoresis. Subsequent electrophoresis of these isozymes in a second dimension by sodium dodecyl sulfate slab gel electrophoresis yielded identical protein patterns for the isozymes with one major protein band at molecular weight 97,000. Malate and AMP were slightly inhibitory (about 20%) to the partially purified enzyme. Phosphoenolpyruvate carboxylase comprised approximately 1 to 2% of the total soluble protein in actively N_2-fixing alfalfa nodules.

Recent studies have demonstrated that nodules of several legume species actively fix CO_2 via the enzyme PEPC[2] (EC 4.1.1.31) and that PEPC may increase nodule carbon use efficiency by recycling a portion of the CO_2 lost through nodule respiration (1, 2, 7, 20). In alfalfa, nodule CO_2 fixation is highly correlated with active N_2-fixation and has been shown to contribute up to 25% of the carbon required for the assimilation of symbiotically derived N (20). Both *in vitro* PEPC activity and *in vivo* CO_2 fixation activity in alfalfa nodules decreased following applications of NO_3^- (20).

Although PEPC has been purified and characterized from nodules of the annual legumes soybean and lupine, regulation of the enzyme activity is not well understood (1, 13). Soybean nodule PEPC activity appears to be regulated by PEP and HCO_3^- concentrations (13). The soybean enzyme was resolved into five isozymes (13). Whereas the soybean enzyme was not appreciably inhibited by addition of either L-aspartate or isocitrate at 1 mM concentrations, lupine nodule PEPC was inhibited 40% by L-aspartate and 60% by isocitrate (1, 13). Lupine nodule PEPC was similar to root and leaf PEPC in kinetic parameters (1). No isozymes were resolved in lupine. Neither NO_3^- nor NO_2^- were evaluated as affectors of PEPC activity in these studies.

Since *in vitro* nodule PEPC activity and *in vivo* CO_2 fixation in alfalfa are inhibited by application of NO_3^- and since nodule PEPC has not been characterized in a perennial legume, we thought it important to characterize PEPC from alfalfa. The objectives of this study were to purify partially PEPC from alfalfa nodules, to study isozyme patterns and some kinetic properties, and to assess the effect of NO_3^- and NO_2^- on purified enzyme activity.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L. cv Saranac) nodules were collected from plants grown in the greenhouse in sandbenches under nilnitrate conditions with supplemental light as described previously (19). Nodules were stored at −20°C until used.

Enzyme Assays. Rates of PEPC activity were measured during the purification procedure using the coupled spectrophotometric assay system described previously (20). Exogenous MDH activity was included in the assay mixture following the hydroxylapatite chromatography step in order to achieve linear rates of reaction.

A radiometric assay similar to that described by Christeller *et al.* (1) was used for determination of the kinetic constants. The standard assay was performed at 24°C in 100 mM Hepes buffer (pH 7.5) containing 5 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (1 μCi, 52 mCi/mmol; ICN)[3], and 2 mM PEP in a total volume of 475 μl. The reaction was initiated by the addition of 25 μl of enzyme solution and terminated after 10 min with 0.3 ml of 6 N HCl. The acid-stable radioactivity was determined by LSC. Reaction rates were linear for at least 15 min.

MDH activity was measured spectrophotometrically, monitoring absorbance due to NADH at 340 nm. The assay contained 50 mM oxaloacetate and 100 μM NADH in 100 mM K-phosphate (pH 7.6) containing 0.1% (v/v) 2-mercaptoethanol.

Enzyme Purification. All procedures were carried out at 0 to 4°C. The buffer, except where indicated, was 20 mM K-phosphate (pH 7.8) containing 10% ethylene glycol and 100 mg/l DTT. The enzyme solution was concentrated by $(NH_4)_2SO_4$ precipitation (65% saturation at 0°C) and the pellet suspended in 20 mM phosphate buffer following each stage of the purification. All chromatography columns were equilibrated in 20 mM phosphate buffer.

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[2] Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; MDH, malate dehydrogenase.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by either the United States Department of Agriculture or approval to the exclusion of other products that may also be suitable.

Fourteen and one-half grams of frozen alfalfa nodules were ground in a cold mortar with 18 g glass beads (0.4–0.5 mm diameter) and 2.9 g wet weight PVP (in extraction buffer) in 30 ml of 100 mM K-phosphate (pH 7.5) containing 25% ethylene glycol and 200 mg/l DTT. The homogenate was centrifuged at 26,200*g* for 25 min to remove cell wall debris and intact bacteroids and the supernatant was collected. The pellets were resuspended in 25 ml of extraction buffer and centrifuged as above. The supernatants were combined, fractionated with solid $(NH_4)_2SO_4$, and the fraction from 50 to 70% saturation was collected and resuspended in a minimum volume of 20 mM phosphate buffer. The enzyme suspension was then applied to the top of a 9.6- × 1.5-cm column of DEAE-cellulose, and PEPC activity was eluted with 20 mM phosphate buffer. Fractions (1.0 ml) containing the highest activity were combined and concentrated. The enzyme suspension was then added to a 50-ml centrifuge tube containing a suspension of 5 ml (settled volume) of hydroxylapatite (Bio-Gel HTP DNA grade, Bio-Rad Laboratories)[3] equilibrated in 20 mM phosphate buffer. The mixture was allowed to sit for 10 min and then was washed with several rinses of 60 mM phosphate buffer (115 ml total volume) by repeated suspension of the hydroxylapatite in 10 to 25 ml of buffer followed by centrifugation at 100*g* for 1 to 2 min and removal of the supernatant. The rinses were continued until little or no MDH activity was spectrophotometrically detectable in the supernatant. The hydroxylapatite was then poured into a small column and washed successively with 60 mM phosphate buffer (10 ml), 70 mM phosphate buffer (15 ml), and 80 mM phosphate buffer (9 ml). PEPC activity was eluted from the column with 240 mM phosphate buffer and fractions (0.5 ml) containing the highest activity were collected. The concentrated enzyme suspension was then further purified by application to a mixed bed column (1:1, v/v) of reactive blue 2-agarose and reactive red 120-agarose (0.4 ml settled volume, Sigma)[3] which had been pretreated with 10 mg BSA/ml of agarose (overnight) followed by extensive rinsing with 0.4 M KCl. The enzyme was eluted with 20 mM phosphate buffer, and 0.25-ml fractions containing the highest PEPC activity were concentrated and stored at −57°C until used for the determination of kinetic constants.

Gel Electrophoresis. Native PAGE was performed using the standard Tris-glycine system of Ornstein and Davis (3, 12) with the addition of 10% and 5% ethylene glycol (v/v, final concentrations) to the running gel (5% crosslinker) and spacer gel, respectively. PEPC activity was located by incubation of the gels according to Scrutton and Fatebene (15) in 100 mM Tris-HCl (pH 8.50 containing 5 mM PEP, 10 mM $MgCl_2$, and 20 mM $NaHCO_3$ for 5 to 15 min followed by a water rinse and dark incubation (5–10 min) in a solution of Fast Violet B (1 mg/ml water). Staining for PEPC activity was reduced by 90% when ethylene glycol was absent from the gel system.

A two-dimensional electrophoretic system was used to investigate the subunit composition of PEPC. Identical samples were run (in 10- × 0.6-cm tubes) in the first dimension as described above. One gel was stained for PEPC activity and was used as a template to slice the duplicate gel into three segments for electrophoresis in the second dimension. The gel segments were frozen overnight and then thawed and individually incubated in three changes of an equilibration buffer (50 ml 0.5 M Tris-HCl [pH 6.7], 20 ml glycerol, 10 ml 2-mercaptoethanol, and 4 g SDS in 120 ml total volume) for a total of 45 min. The slices were then placed on top of a 0.75-mm thick SDS slab gel consisting of a 10% running gel (12.5 cm) and a 3% spacer gel (3 cm) and cemented in with warm 1% agarose (9). A series of mol wt standards were also run into the second dimension gel. Approximately 4 μg of each of the following standard proteins were polymerized into a 0.8- × 0.6-cm piece of native running gel which was then treated similarly to above gel segments following their electrophoresis in the first dimension: β-galactosidase (138,000), phosphorylase b (94,000), BSA (68,000), creatine phosphokinase (40,000), alcohol dehydrogenase (35,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), RNase (14,000), and Cyt *c* (12,800). The slab gel was stained with Coomassie brilliant blue-R for protein identification.

Soluble protein was measured according to the procedure described by Lowry *et al.* (9).

All values for kinetic constants were the average of two to four separate enzyme preparations. K_m values were calculated from regression analyses of Eadie-Hofstee plots. The K_i value for oxaloacetate was calculated from Lineweaver-Burk plots of data obtained when PEP carboxylation was allowed to proceed at three different concentrations of oxaloacetate.

RESULTS AND DISCUSSION

Enzyme Purification. Alfalfa root nodule PEPC was purified 86-fold with a final yield of 32% with respect to the crude extract (Table I). The preparation was judged to be 70% pure by PAGE. The major contaminating protein appeared to be MDH, an abundant alfalfa nodule enzyme composed of several isozymes (data not shown). The major portion of MDH could be removed by the hydroxylapatite and reactive agarose steps. Pretreatment of reactive agarose with BSA (see "Materials and Methods") was essential to control losses of PEPC in that purification step. Without the BSA pretreatment, there was a 100% loss of PEPC activity. There was a nonspecific binding between the enzymes and untreated reactive agarose gel. It should be noted that MDH activity was substantially reduced after the hydroxylapatite step and exogenous sources of MDH were added to the enzyme assay to obtain linear activity.

A pH of 7.8 phosphate buffer in the DEAE-cellulose step was needed to prevent binding of the enzyme to the column material and subsequent losses of up to 70% of enzyme activity. This pH is somewhat higher than that used in (pH 7.0–7.2) purifications of PEPC from other sources (1, 13, 16).

PH Optimum and Substrate Kinetics. The pH optimum for purified alfalfa nodule PEPC was 7.5 with over half maximum activity retained between pH 7.0 and 9.0. This value is similar to that reported for the lupine root nodule enzyme (1).

Some inhibition of enzymatic activity (26% as compared to standard Hepes buffer) was noted when assays were performed in phosphate buffer (100 mM, pH 7.5). Inhibition of PEPC by phosphate buffers has also been noted by Miziorko *et al.* (10) for spinach leaf PEPC and by Peterson and Evans (13) for soybean nodule PEPC when phosphate was used in the electrophoresis system.

At pH 7.5, alfalfa nodule PEPC showed hyperbolic kinetics with respect to both PEP and Mg^{2+}, with apparent K_m values of 210 and 100 μM, respectively. The K_m for PEP for the alfalfa nodule enzyme is approximately twice the value reported for the

Table I. *Partial Purification of Phosphoenolpyruvate Carboxylase from Alfalfa (Medicago sativa) Root Nodules*

Purification Step	Protein	Total Units[a]	Specific Activity	Recovery
	mg		*units/mg protein*	*%*
Crude extract	227	111	0.49	100
$(NH_4)_2SO_4$, 50–70%	69.5	104	1.50	94
DEAE-cellulose	21.9	47.4	2.16	43
Hydroxylapatite	2.18	35.9	16.5	32
Reactive agarose	0.84	35.5	42.3	32

[a] One unit of activity is defined as that forming 1 μmol of product/min.

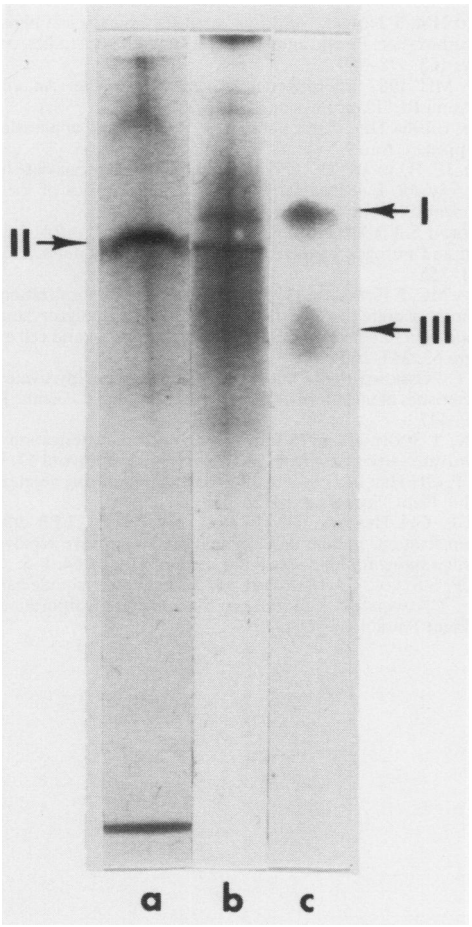

FIG. 1. Electrophoresis of alfalfa nodule PEPC purified through the reactive agarose step (Table I) on a nondenaturing 5% polyacrylamide slab gel. All lanes shown were cut from a single slab gel and stained for either MDH activity (lane a), protein (lane b), or PEPC activity (lane c). Lanes a, b, and c contained 15.6, 13.0, and 7.8 μg protein, respectively (I and III = positive-staining band for PEPC, II = positive-staining band for MDH).

lupine and soybean root nodule enzymes (1, 13). The apparent K_m for Mg^{2+} was determined in the presence of saturating levels of PEP and is similar to values reported for the lupine nodule enzyme (1). Substitution of 20 mM $MnCl_2$, $CaCl_2$, and $ZnCl_2$ in the assay for 20 mM $MgCl_2$ resulted in 90 to 100% inhibition of enzyme activity. Although we could not remove all of the HCO_3^- from the reagents and the enzyme preparation, we estimated the K_m for HCO_3^- to be between 100 and 400 μM, within the range of that reported for the soybean nodule enzyme (13).

Inhibition of Enzyme Activity. Several metabolites were tested for their effect on PEPC activity. The following metabolites were included in the standard radiometric assay at 1 mM final concentration and had no effect except where discussed: citrate, isocitrate, succinate, malate, malonate, pyruvate, α-ketoglutarate, fumarate, oxaloacetate, glycine, L-aspartic acid, L-asparagine, DL-glutamic acid, L-glutamine, DL-alanine, ATP, ADP, AMP, NADH, NAD, NADPH, NADP, KNO_3, $NaNO_2$, and NH_4Cl. Inhibition of PEPC was observed when oxaloacetate was added to the assay (48% inhibition at 1 mM) with a K_i of 9.6 mM. Slight inhibition was observed with malate (18%) and AMP (16%). Inhibition of PEPC activity by oxaloacetate, malate, and AMP have been reported previously (11). Inhibition observed with oxaloacetate is probably not of physiological significance since *in vivo* oxaloacetate concentrations are low (10).

Addition of NO_2^- to the standard radiometric assay appeared to inhibit PEPC activity. However, rather than inhibition of enzyme activity, the NO_2^- addition caused nonenzymic decarboxylation of oxaloacetate, the product of PEPC, resulting in a loss of ^{14}C-labeled carbon. Inclusion of MDH and NADH in the radiometric assay catalyzed rapid conversion of [^{14}C]oxaloacetate to [^{14}C]malate and showed no loss of activity resulting from NO_2^-. Although NO_2^- does accumulate in nodules of NO_3^--treated plants, nonenzymic decarboxylation of oxaloacetate by NO_2^- is likely not physiologically significant because of high MDH activity in nodules.

Evidence for Isozymes and Subunit Composition. The partially purified enzyme was electrophoresed in a nondenaturing system and the gel stained for either protein, PEPC activity, or MDH activity (Fig. 1). Protein staining showed two distinct bands (I and II) and one broad band III, while enzyme staining revealed two isozymes of PEPC activity corresponding to protein bands I and III and one remaining isozyme of MDH corresponding to protein band II. Occasionally, protein band II also exhibited PEPC activity when increased quantities of protein were loaded in the gel or when gels were incubated in the substrate for long periods of time. These results indicate there are at least two isozymes of PEPC in alfalfa nodules. Similar results have been obtained with crude preparations of alfalfa root nodules (4). In contrast, Peterson and Evans (13) reported that soybean nodule PEPC could be separated into five isozymes using an imidazole PAGE system. Protein band II in our system may represent a mixture of MDH and PEPC.

To investigate the subunit composition of alfalfa nodule PEPC, tube gel sections corresponding to protein bands I, II, and III were electrophoresed into a second dimension of SDS polyacrylamide slab gel. Second dimension electrophoresis of protein bands I and III, which stain for PEPC activity, showed identical protein patterns consisting of one intense protein band (about mol wt 97,000) and two very faint bands. These results suggest that alfalfa root nodule PEPC is composed of one or more identical subunits and the subunit mol wt (about 97,000) of the two isozymes is likely identical.

Electrophoresis of protein band II in the second dimension yielded more complex protein pattern than bands I and III. It included the intense protein band at mol wt 97,000 similar to that identified in the two dimensional pattern of bands I and III as well as several smaller (<35,000) mol wt proteins. The appearance of the 97,000 mol wt protein in band II supports the observation that PEPC may be present in band II. It is not clear if band II represents a third isozyme of PEPC or whether one of the two identifiable PEPC isozymes had altered migration. The smaller mol wt proteins likely represent subunits of MDH.

The estimated monomer mol wt of alfalfa nodule PEPC of 97,000 based on migration in SDS polyacrylamide gels is similar to monomer subunit weights of maize leaf PEPC (100,000) and *Bryophyllum fedtschenkoi* (100,000), both sources in which the enzyme is believed to be a tetramer of identical monomers (6, 17). Mol wt estimations have not been reported for either soybean or lupine nodule PEPC. No attempts were made in this study to determine the mol wt of the alfalfa root nodule PEPC holoenzyme.

Based on the purification data presented here and assuming a 70% purity for the preparations, it is estimated that PEPC comprises approximately 1 to 2% of the total soluble protein in actively N_2-fixing alfalfa nodules. CO_2 fixation via nodule PEPC has been reported to provide up to 25% of the carbon required for assimilation and transport of symbiotically fixed N in alfalfa (20). Studies of nodule PEPC presented here further underline the importance of this enzyme in root nodule carbon and nitrogen metabolism.

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