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Alfalfa Root Nodule Carbon Dioxide Fixation[1]

III. IMMUNOLOGICAL STUDIES OF NODULE PHOSPHOENOLPYRUVATE CARBOXYLASE

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ABSTRACT

Antiserum was prepared in rabbits against purified alfalfa (*Medicago sativa* L.) nodule phosphoenolpyruvate carboxylase (PEPC). Immunotitration assays revealed that the antiserum recognized the enzyme from alfalfa nodules, uninoculated alfalfa roots, and from soybean nodules. Tandem-crossed immunoelectrophoresis showed that the PEPC protein from alfalfa roots and nodules was immunologically indistinguishable. The 101 kilodalton polypeptide subunit of alfalfa nodule PEPC was identified on Western blots. The PEPC polypeptide was detected in low quantities in young alfalfa roots and nodules but was present at increased levels in mature nodules. Senescent nodules appeared to contain a reduced amount of the PEPC polypeptide. PEPC was also detected by western blot in some plant- and bacterially-conditioned ineffective alfalfa nodules but was not detected in bacteroids isolated from effective nodules. Alfalfa nodule PEPC is constitutively expressed in low levels in roots. In nodules, expression of PEPC polypeptide increases several-fold, resulting in increased PEPC activity. Antiserum prepared against the C_4 PEPC from maize leaves recognized the PEPC enzyme in all legume nodules and roots tested, while the antiserum prepared against alfalfa nodule PEPC also recognized the leaf PEPC of several C_4 plant species. Neither antiserum reacted strongly with any C_3 leaf proteins. The molecular weight of the PEPC polypeptide from C_4 leaves and legume nodules appears to be similar.

Rhizobium-legume induced root nodules are highly organized hyperplastic tissue masses derived from root cortical cells (21). Root nodule formation and function requires numerous specific events which are controlled by both the host plant and *Rhizobium* genomes (21). While substantial progress has been made in identifying rhizobial genes and gene products that regulate symbiosis, relatively little progress has been made in understanding the contribution of plant genes to symbiosis (2, 28). Several symbiotic genes have been documented through classical genetic studies of pea *Pisum sativum* L. (2), clover *Trifolium pratense* L. (15), soybean *Glycine max* L. Merr. (29), chickpea *Cicer arietinum* L. (5), and alfalfa *Medicago sativa* L. (18), yet the physiological and biochemical manifestation of these genes is poorly understood.

Studies with soybean (11, 28), pea (2), and alfalfa (10, 23) showed that host plant genes code for some 20 to 30 nodulins.[2] These are plant proteins found only in root nodules and not in roots or other plant organs (27). Nodulin expression has been shown to be a function of the stage of nodule development and dependent upon the effectiveness of the symbiosis (2, 10, 23, 28). Although the function of most nodulins remains unclear, three have been identified. Leghemoglobin is the classic example of a nodulin which has been characterized from numerous species (28). More recently uricase and GS (EC 6.3.1.2) from nodules of soybean and common bean *Phaseolus vulgaris* L., respectively, have been identified as nodulins (1, 4).

In alfalfa, the activity of PEPC (EC 4.1.1.31), GOGAT (EC 1.4.1.14), and GS is substantially higher in actively N_2-fixing nodules than in roots (7, 26). Treatments that reduce N_2 fixation also cause a concomitant reduction in PEPC, GOGAT, and GS activities (26). In both plant- and rhizobial-controlled ineffective nodules, PEPC, GOGAT, and GS activities are reduced by 60 to 90%. Using Western blots, our laboratory recently documented the presence of at least 19 nodulins in alfalfa (23). Many of the nodulins observed from effective nodules were either substantially reduced or absent in ineffective nodules. Some of these alfalfa nodulins probably correspond to PEPC, GOGAT, and GS.

Alfalfa root nodule PEPC contributes as much as 25% of the C required for the assimilation of fixed N (13, 26). The enzyme comprises about 1 to 2% of the total soluble protein in actively N_2-fixing nodules. The specific activity of alfalfa nodule PEPC is 6- to 12-fold that in roots (25). These data support the suggestion (23) that alfalfa nodule PEPC is a nodule specific protein. However, they do not rule out the possibility that PEPC is a protein that occurs in roots which is highly enhanced in effective nodules. The objectives of this study were: (a) to prepare antibodies to root nodule PEPC, (b) to utilize immunological methods to ascertain if PEPC is a nodulin, (c) to assess if a protein similar to alfalfa nodule PEPC is conserved in nodules of other legume species, and (d) to ascertain if antibodies to nodule PEPC recognized the PEPC polypeptide in leaves of C_4 species.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L. cv Saranac), birdsfoot trefoil (*Lotus corniculatus* L. cv Norcen), adzuki bean (*Vigna angularis* Willd, Ohioi and Ohashi, cv Takara), soybean (*Glycine max* L. Merr. cv Hodson), pea (*Pisum sativum* L. cv Alaska), Texas bluebonnet (*Lupinus subcarnosus* L. cv Hook), and clover (*Trifolium pratense* L. cv Lakeland) were grown in

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[2] Abbreviations: nodulins, nodule specific proteins; PEPC, phosphoenolpyruvate carboxylase; GS, glutamine synthetase; GOGAT, glutamate synthase; PMSF, phenylmethylsulfonylfluoride.

the glasshouse as previously described (22, 23). Host-conditioned ineffective plants *in*$_1$ag (Agate background), *in*$_1$sa (Saranac background), and *in*$_2$ and *in*$_3$ (both tumor-like) were produced on clonally propagated plants as described by Peterson and Barnes (18). Bacterially conditioned ineffective plants were grown in enclosed culture tubes on agar. The appropriate *Rhizobium meliloti* inoculum[3] was applied at the time of seeding (Nitragin Co., Milwaukee, WI; and Sharon Long, Stanford University, CA) for all plant species. Nodules were picked at various developmental stages and frozen at −20°C until used.

Sorghum (*Sorghum bicolor* L.), redroot pigweed (*Amaranthus retroflexus* L.), wheat (*Triticum aestivum* L. cv Marshall), and maize (*Zea mays* L.) plants were grown in pots in a glasshouse in unsupplemented soil. Leaves for protein extraction were removed from the plants 2 to 4 weeks following planting and frozen at −80°C until used.

Antiserum Production. Alfalfa nodule PEPC was purified as previously described (25). The preparation was then electrophoresed on a preparative SDS polyacrylamide gel, and the protein band corresponding to the polypeptide subunit of PEPC was excised from the gel and frozen at −20°C until used for injections. The purity of the excised band was checked by silver staining (23). The mol wt of the excised band was confirmed by western blot with alfalfa whole nodule antiserum (23) after elution of the polypeptide from the excised band and electrophoresis in a second SDS gel. Injections were prepared by pulverizing a portion of the frozen gel in liquid N_2 in a mortar and pestle. The powdered gel was then resuspended in PBS buffer (0.05 M sodium phosphate [pH 7.4] containing 0.9% NaCl) and mixed with adjuvant for injection of rabbits (New Zealand White). All immunizations were subcutaneous with Freund's complete adjuvant used in the initial injection and incomplete adjuvant used in the four subsequent injections scheduled at 10 to 15 d intervals. A total of 1.5 mg of PEPC protein was injected. Rabbits were bled 10 to 13 d after each of the final two injections. Blood serum was concentrated and titer determined on Ouchterlony plates as previously described (23). Antiserum raised in rabbits against maize leaf PEPC was a gift from Dr. R. Chollet, University of Nebraska.

Immunotitration of PEPC Activity. Freshly harvested nodules from 30-d-old alfalfa, 28-d-old soybean, and uninoculated roots and root tips of alfalfa sprouts were extracted in 20 mM potassium phosphate (pH 7.8) containing 10% ethylene glycol and 100 mg DTT/L. Antipain and PMSF were included in all extractions at final concentrations of 10 μM and 1 mM, respectively. A constant quantity of protein from each source was incubated for 1.5 h at 4°C with varying quantities (25–250 μl) of PEPC antiserum. Immune complexes formed were precipitated by the addition of 90 μg of goat anti-rabbit IgG (Sigma Chemical Co.) followed by an additional incubation for 1.5 h at 4°C. The PEPC activity remaining in the supernatant following centrifugation at 15,000*g* for 20 min was measured by radiometric assay (25). Rabbit serum protein in each incubation (based on Lowry [12] assay) was made equivalent by the addition of preimmune rabbit serum. A 250 μl preimmune serum control incubation was also run for each protein source.

Western Blotting. Protein from nodule and root samples was prepared as previously described (23). Proteins were extracted from leaves by grinding 1 g fresh weight of leaf tissue (cut into 2-cm pieces with a razor blade) in a prechilled mortar and pestle in 4 ml per g fresh weight of cold grinding medium including protease inhibitors. The grinding medium consisted of 30 mM Tris-Mes (pH 7.5) containing 0.5 M sucrose, 5 mM EDTA, 5 mM DTT, and 0.5% PVP (insoluble). Antipain and PMSF were added at final concentrations of 20 μM and 2 mM, respectively. The homogenates were centrifuged at 16,800*g* for 25 min and the supernatants were collected and recentrifuged as above. The supernatants from the second centrifugation were frozen at −80°C until used for electrophoresis. Protein samples of 5 to 50 μg were separated in 10% SDS-PAGE with electrophoretic transfer of the proteins to nitrocellulose as described previously (23). Proteins of interest were visualized with PEPC antiserum as described previously (23). Mol wt markers consisted of phosphorylase B (94 kD), BSA (68 kD), and creatine phosphokinase (40 kD). Marker proteins on nitrocellulose were visualized by India ink staining as described by Hancock and Tsang (9). Protein was quantitated by the method of Lowry *et al.* (12) using 10% TCA precipitates.

Bacteroid Protein. Alfalfa bacteroids and peribacteroid proteins were isolated according to the procedure described by Bisseling *et al.* (2). The bacteroids were lysed by resuspension in a buffer consisting of 67 mM Tris-Cl (pH 6.7) containing 4% SDS, 2% mercaptoethanol, and 2 mM PMSF followed by heating to 80°C for 3 min. The bacteroid protein solution was then clarified by centrifugation at 14,000*g* for 20 min. Protein assays were performed on acetone precipitates by the method of Lowry *et al.* (12).

Immunoelectrophoresis. Tandem-crossed immunoelectrophoresis was carried out using nodule PEPC antiserum. The first dimension gel consisted of 1% agarose in Tris-barbital (pH 8.6). Well No. 1 contained either 124 μg of alfalfa root protein or 46 μg of alfalfa nodule protein, each fractionated between 50 to 65% saturation with $(NH_4)_2SO_4$. Well No. 2 contained 2 μg of partially purified alfalfa nodule PEPC. Electrophoresis was carried out for 1.5 h at 10 V/cm followed by transfer of the first dimension lane to a clean glass plate. A 1% agarose gel containing 2% PEPC antiserum (final concentration) was cast adjacent to the first dimension lane. Electrophoresis was carried out overnight at 2 V/cm in a direction perpendicular to the first dimension. Gels were dried onto Gel Bond film (FMC Corp., Rockland, ME) and the immunoprecipitate was stained with Coomassie brilliant blue.

RESULTS

Immunotitration of PEPC activity in crude extracts with nodule anti-PEPC serum showed that enzyme activity was inhibited in all root and nodule sources (Fig. 1). The shape of the titration curves reflects the PEPC specific activity of each tissue and the amount of PEPC protein in each extract. The amount of serum required to titrate PEPC activity from roots, root tips, and nodules was proportional to the enzyme activity of each tissue. Nearly complete titration of root and root tip PEPC activity was achieved with 100 μl of serum while alfalfa root nodules retained 80% of the initial PEPC activity. As demonstrated previously (13) alfalfa root PEPC activity was considerably lower than that of root nodules. Consistent with previous studies (22), soybean nodule PEPC specific activity was lower than that of alfalfa nodules. Soybean PEPC activity was reduced by 93% with the addition of 200 μl of PEPC serum. Immunotitration with preimmune serum had no effect on PEPC activity from any source (data not shown).

Tandem-crossed immunoelectrophoresis of soluble cytoplasmic proteins from uninfected roots and effective root nodules of alfalfa with partially purified nodule PEPC run as a reference and using nodule anti-PEPC serum resulted in double-fused precipitin peaks in both instances (Fig. 2, A and B) showing that root and nodule PEPC were immunologically indistinguishable.

The developmental profile of PEPC in alfalfa nodules was evaluated using western blots (Fig. 3). Alfalfa seedlings were

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by either the U.S. Department of Agriculture or the University of Minnesota and does not imply its approval to the exclusion of other products that may also be suitable.

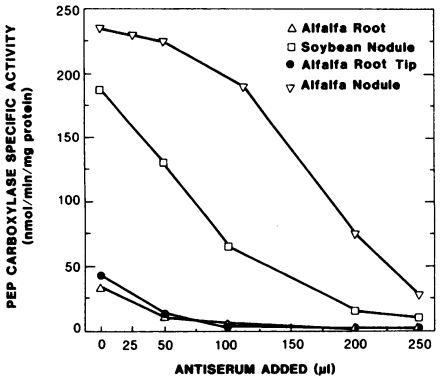

FIG. 1. Immunotitration of PEPC activity from alfalfa nodules (▽, 420 µg protein), soybean nodules (□, 450 µg protein), alfalfa roots (△, 400 µg protein), and alfalfa root tips (●, 400 µg protein) using antiserum prepared against alfalfa nodule PEPC. A constant quantity of protein from each source was incubated with anti-PEPC serum (25–250 µl). Goat anti-rabbit IgG (90 µg) was added to each reaction to precipitate the immune complexes and the activity of PEPC remaining in the supernatant following centrifugation was assayed radiometrically. Preimmune serum (250 µl) was incubated with proteins from each source as a control.

inoculated with *R. meliloti* at the unifoliate leaf stage, and root or nodule tissue was taken 6, 9, 13, 22, and 36 d after inoculation. On day 36, plant tops were cut back to simulate forage grazing, and 7 d later nodules were collected. Comparable amounts (15 µg) of crude soluble cytoplasmic protein from each day were separated by SDS-PAGE and probed with nodule anti-PEPC serum.

Six d after inoculation no visible nodules were present; therefore, roots were extracted for analysis. A slight amount of the PEPC polypeptide at 101,000 mol wt (the subunit mol wt of PEPC) was already evident (Fig. 3). By d 9, very small, white nodules were visible on the roots. These white nodules, similar to roots, had only slight amounts of the PEPC polypeptide. As nodule size increased and pink color developed at 13 d following inoculation, the PEPC polypeptide became more evident. The PEPC polypeptide increased through 36 d following inoculation as demonstrated by the increased band intensity. Nodules sampled 7 d after removing plant tops were partially senescent as previously reported (26). While these partially senescent nodules contained the PEPC polypeptide, the decreased intensity of the 101 kD band indicated that the quantity of PEPC was reduced compared to that found on d 36.

The PEPC specific activity ($\mu mol \cdot min^{-1} \cdot g$ fresh $weight^{-1}$) of d 6, 9, 13, 22, 36 and senescent root and nodule tissue was 0.08, 0.78, 2.29, 5.02, 9.40, and 5.51, respectively. Similar to immunotitration studies (Fig. 1), the size and intensity of the PEPC band detected on Western blots were directly proportional to *in vitro* enzyme activity.

The PEPC polypeptide was detected in ineffective nodules controlled by the plant gene in_1 but not in ineffective nodules controlled by the plant genes in_2 and in_3 (Fig. 4). A cross-reactive protein at 101 kD mol wt was also detected in bacterially conditioned ineffective alfalfa nodules formed by two different ineffective *Rhizobium* strains (Fig. 4). Consistent with previous studies that showed a 5- to 7-fold reduction of PEPC activity in ineffective nodules (24, 26), detection of the PEPC polypeptide on a Western blot required a 10-fold increase in the quantity of ineffective nodule protein as compared to effective nodule protein. A polypeptide equivalent to effective nodule PEPC also was not detected in either the peribacteroid or bacteroid fractions isolated from actively N_2-fixing alfalfa nodules when 50 µg of protein from each source was electrophoresed.

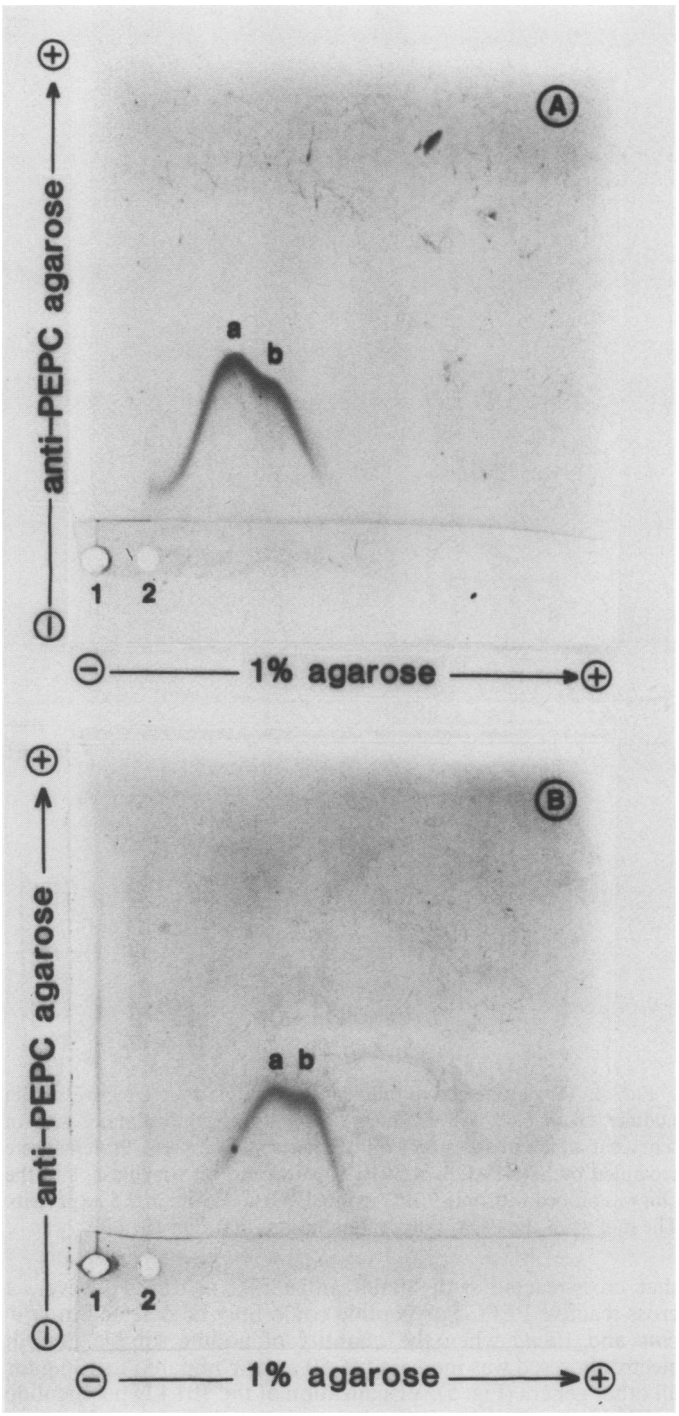

FIG. 2. Tandem-crossed immunoelectrophoresis of the 50 to 65% ammonium sulfate fractions of soluble protein from alfalfa nodules (A) and alfalfa roots (B). Application well 1A, alfalfa nodule protein (46 µg protein) giving precipitate a; application well 1B, alfalfa root protein (124 µg protein) giving precipitate a; application wells 2A and B, partially purified alfalfa nodule PEPC (2 µg protein) giving precipitate b. First dimension electrophoresis was into 1% agarose. Second dimension electrophoresis was into 1% agarose containing 2% (v/v) anti-PEPC serum.

To assess if the alfalfa nodule PEPC polypeptide was conserved in other legumes, soluble protein from actively N_2-fixing nodules of seven genera were separated by SDS-PAGE and probed with alfalfa nodule anti-PEPC serum (Fig. 5). Nodules from all of the genera tested contained a polypeptide band of 101 kD mol wt

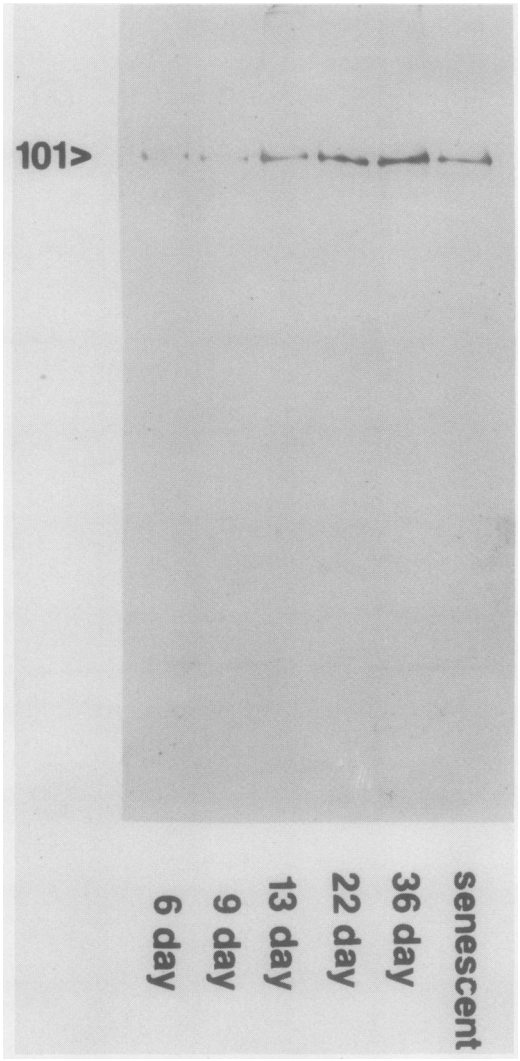

FIG. 3. Developmental profile of PEPC found in effective alfalfa nodules taken 6, 9, 13, 22, and 36 d following inoculations and in senescent alfalfa nodules (taken 7 d after shoot harvest). Proteins were separated by SDS-PAGE, western blotted onto nitrocellulose, and the blot was probed with anti-PEPC serum. Each lane contains 15 μg protein. The mol wt of the PEPC polypeptide was estimated at 101,000.

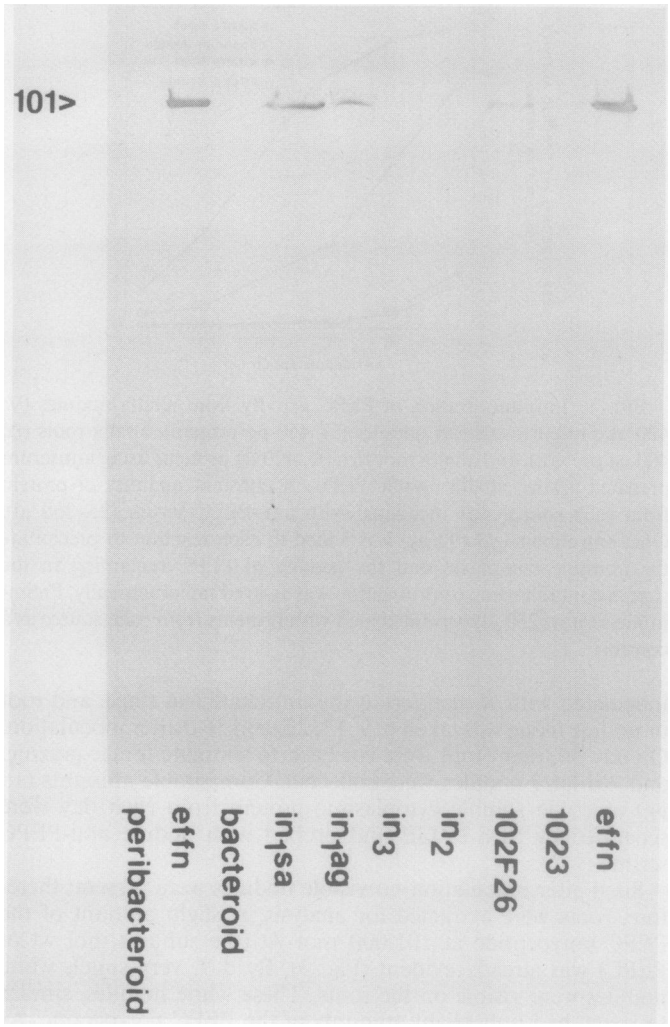

FIG. 4. Western blot of soluble proteins from alfalfa peribacteroid space (peribacteroid), effective alfalfa nodules (effn), effective alfalfa nodule bacteroids (bacteroid) and several plant (in_1sa, in_1ag, in_3, in_2) and bacterially (102F26, 1023) conditioned ineffective alfalfa nodules probed with alfalfa nodule anti-PEPC serum. Proteins were blotted from an SDS-PAGE gel onto nitrocellulose. Lanes 2 and 12 (effn) contain 10 and 15 μg protein, respectively, while all other lanes contain 50 μg protein. The mol wt of the major polypeptide was estimated at 101,000.

that cross-reacted with alfalfa anti-PEPC serum. However, a cross-reactive PEPC polypeptide could only be detected in *Glycine* and *Vigna* when the quantity of nodule soluble protein electrophoresed was increased to 50 μg as compared to 13 μg for all other genera (Fig. 5). Visualization of the 101 kD polypeptide in alfalfa roots also required the electrophoresis of 50 μg of soluble protein.

The soluble nodule proteins from the seven genera of legumes were also probed with antiserum produced against the C_4 PEPC purified from maize leaves in order to compare the cross-reactivity of maize anti-PEPC against nodule PEPC (Fig. 6). The maize leaf anti-PEPC serum strongly reacted with a polypeptide of approximately 101,000 mol wt from maize leaves. The maize leaf anti-PEPC serum also recognized the nodule PEPC polypeptide in all of the legume genera tested as well as the PEPC polypeptide from alfalfa roots. All of the reactive polypeptides were of the same apparent mol wt. The banding patterns and band intensities for each of the genera tested using the two different anti-PEPC serums were identical (*cf.* Figs. 5 and 6), including some minor, lower mol wt polypeptides that are likely PEPC breakdown products (see "Discussion").

The antiserum produced against the C_4 PEPC from maize leaves was also used to probe a blot containing soluble proteins from leaves of various C_3 and C_4 species and alfalfa nodules and root to determine the cross-reactivity of the C_3 and C_4-type PEPC polypeptides (Fig. 7). A large, intensely staining band at 100,000 mol wt, the subunit mol wt of PEPC, was evident when 5 μg of maize leaf protein was electrophoresed. Strongly cross-reactive polypeptides of the same mol wt as the maize leaf polypeptide were also observed when the soluble leaf proteins from the C_4 species *Sorghum* and *Amaranthus* were electrophoresed. In contrast, slight to no reaction was observed when the soluble leaf proteins from the C_3 species *Triticum* and *Medicago* were probed with the maize leaf anti-PEPC serum, in spite of the fact that the protein load for the C_3 species was increased over that used for the C_4 species (Fig. 7 legend). Similar to the leaves of C_4 species, the PEPC polypeptide of *Medicago* nodules and roots cross-reacted strongly with the maize leaf anti-PEPC serum (Fig. 7).

The nodule anti-PEPC serum was also used to probe a blot containing the soluble leaf proteins of the C_3 and C_4 species

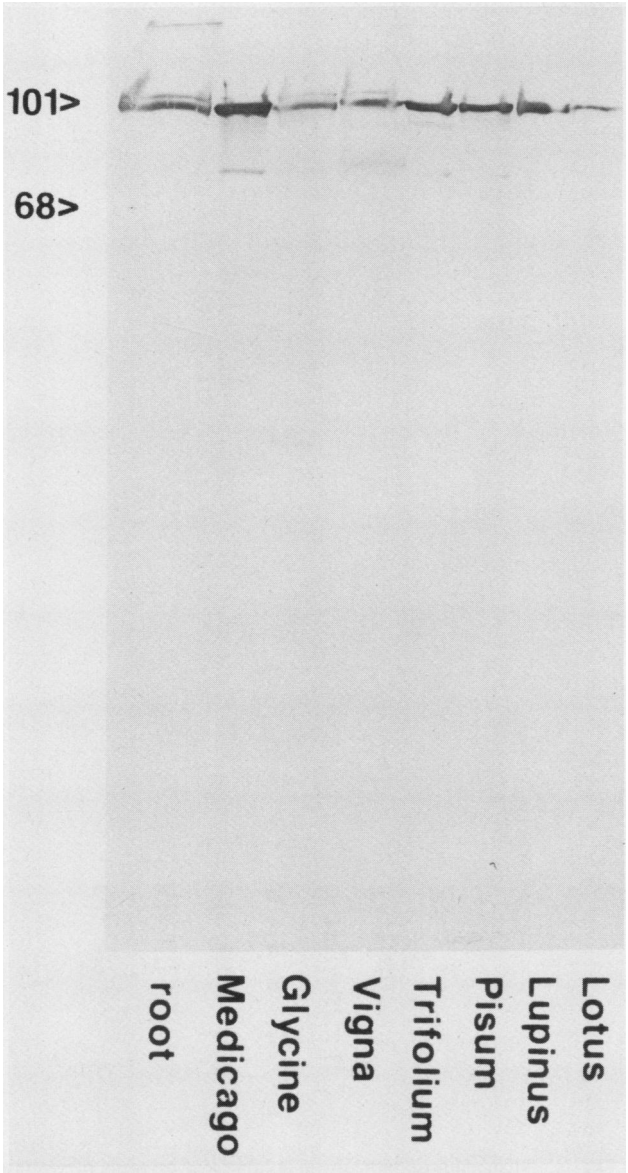

FIG. 5. Cross-reactivity of alfalfa nodule anti-PEPC serum with effective nodule protein of various legume species. Nodule soluble proteins and root proteins were electrophoresed in an SDS-PAGE system, the proteins were transferred to nitrocellulose and the blot was probed with alfalfa nodule anti-PEPC serum. Each lane contains 13 μg nodule soluble protein except the *Glycine*, *Vigna*, and root lanes which contain 50 μg protein. Lane designations are: root, alfalfa root; alfalfa, *Medicago*; soybean, *Glycine*; adzuki bean, *Vigna*; clover, *Trifolium*; pea, *Pisum*; Texas blubonnet, *Lupinus*; trefoil, *Lotus*.

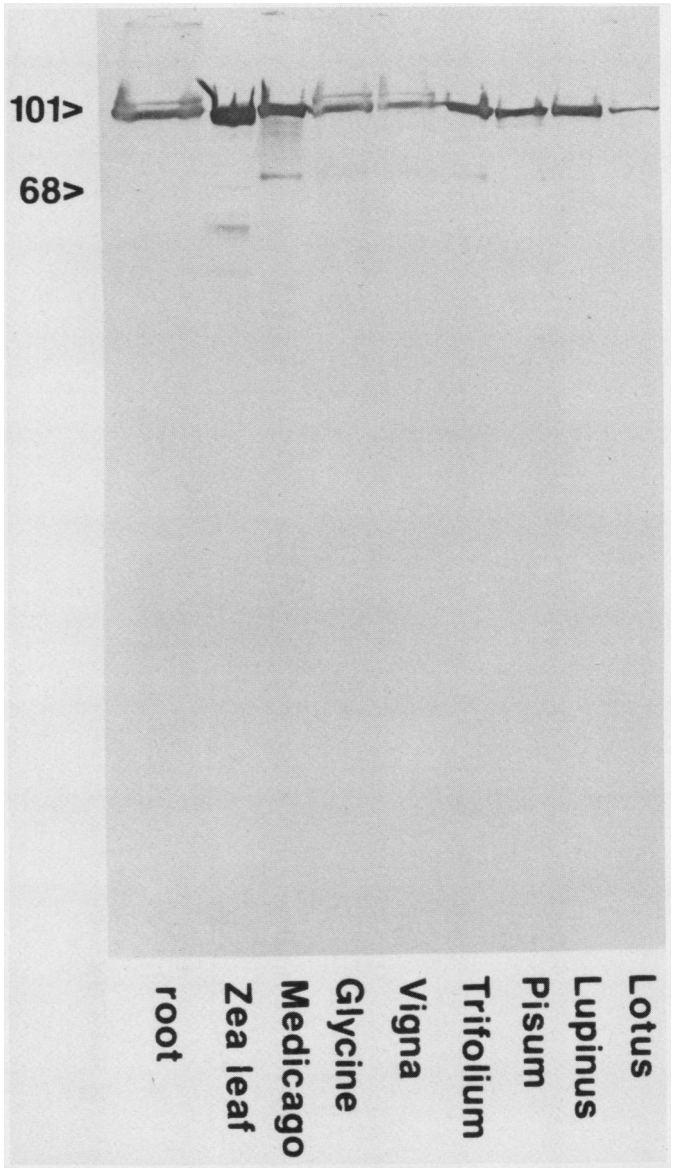

FIG. 6. Cross-reactivity of maize leaf anti-PEPC serum with effective nodule proteins of various legume species. Soluble proteins from maize leaves, alfalfa roots, and nodules of several legume species were electrophoresed in an SDS-PAGE system, the proteins were transferred to nitrocellulose, and the blot was probed with maize leaf anti-PEPC serum. The maize leaf lane contains 5 μg protein while the root, *Glycine* nodule, and *Vigna* nodule lanes contain 50 μg protein. The remaining nodule lanes each contain 13 μg protein. Lane designations are: root, alfalfa root; maize leaf, *Zea* leaf; alfalfa, *Medicago*; soybean, *Glycine*; adzuki bean, *Vigna*; clover, *Trifolium*; pea, *Pisum*; Texas bluebonnet, *Lupinus*; trefoil, *Lotus*.

similar to the blot probed with the leaf anti-PEPC serum seen in Figure 7 in order to compare the reactivity of the two different sera against the leaf PEPC polypeptide (Fig. 8). Probing the blot with the nodule anti-PEPC serum resulted in a banding pattern and band intensities identical to those given by the maize leaf anti-PEPC serum (*cf.* Figs. 7 and 8). Again, the staining of some of the minor, lower mol wt polypeptides was identical when comparing the reaction of the two sera, indicating that those polypeptides are likely breakdown products of PEPC.

DISCUSSION

The antiserum developed against root nodule PEPC was monospecific and highly sensitive as shown by single tandem-crossed immunoelectrophoresis and a single reactive band on Western blots. Positive reactions on Western blots were obtained with as little as 5 μg of crude total nodule soluble protein. Immunological evidence indicates that alfalfa root nodule PEPC and the root PEPC protein are strikingly similar, if not identical. In contrast to leghemoglobin (28), soybean nodule uricase-35 (1) and *Phaseolus* root nodule GS (4) which are nodulins (proteins found only in root nodules and not other plant organs), alfalfa root nodule PEPC is a root protein which is highly enhanced in actively N_2-fixing nodules. Immunotitration, *in vitro* enzymatic activity (25, 27), Western blot band intensity, and rocket im-

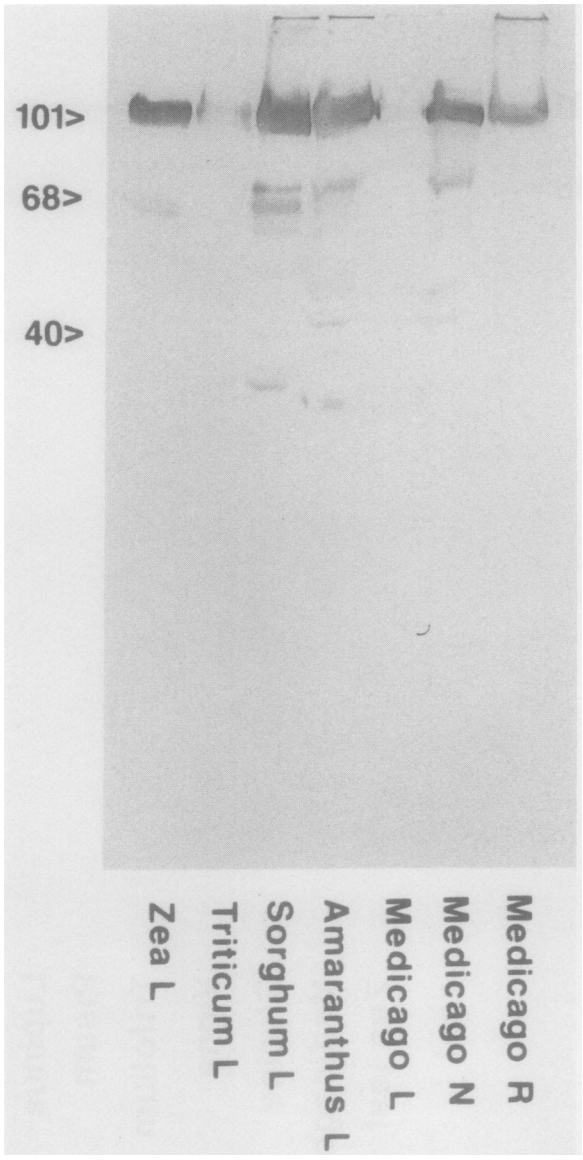

FIG. 7. Nitrocellulose blot of the soluble leaf proteins of various C_3 and C_4 plant species probed with maize leaf anti-PEPC serum. Proteins were electrophoresed in an SDS-PAGE system and then transferred to nitrocellulose. Tissue designations are: L, leaf; N, nodule; R, root. The protein loads are: *Zea* L, 5 μg; *Triticum* L, 40 μg; *Sorghum* L, 10 μg; *Amaranthus* L, 27 μg; *Medicago* L, 40 μg; *Medicago* N, 36 μg; *Medicago* R, 50 μg.

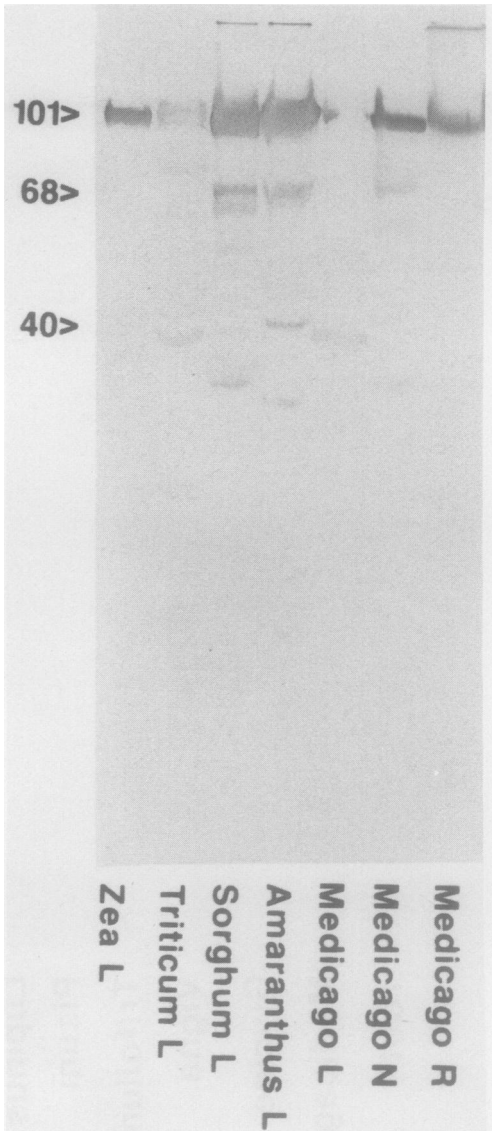

FIG. 8. Nitrocellulose blot of the soluble leaf proteins of various C_3 and C_4 plant species probed with alfalfa nodule anti-PEPC serum. Proteins were electrophoresed in an SDS-PAGE system then transferred to nitrocellulose. The tissue designations are: L, leaf; N, nodule; R, root. The protein loads are: *Zea* L, 5 μg; *Triticum* L, 40 μg; *Sorghum* L, 10 μg; *Amaranthus* L, 27 μg; *Medicago* L, 40 μg; *Medicago* N, 18 μg; *Medicago* R, 50 μg.

munoelectrophoresis (data not shown) indicate that nodule PEPC protein is enhanced 5- to 20-fold as compared to roots. Further evidence for the plant origin of alfalfa root nodule PEPC is the absence of comparable cross-reacting polypeptides in either free-living *Rhizobium meliloti* (data not shown) or bacteroids isolated from actively N_2-fixing nodules.

The enhancement of the PEPC polypeptide in nodules appears to be directly related to the capacity of the nodule to fix N_2. Previous developmental studies in our laboratory (26) have shown that PEPC activity increased in concert with nodule nitrogenase activity and that treatments that reduced nodule nitrogenase activity also reduced PEPC activity. These changes in PEPC activity during nodule ontogeny appear directly related to increases and decreases in the quantity of the PEPC polypeptide (Fig. 3).

Similarly, the low PEPC activity in ineffective nodules (8, 24) can be linked to decreased quantities of the PEPC polypeptide (Fig. 4). Interestingly, the amount of PEPC polypeptide in the ineffective nodules varied according to the cause of ineffectiveness. Nodules that contained sizeable numbers of bacteria and/or bacteroids (in_1sa, in_1ag, 102F26, and 1023) displayed a cross-reactive band corresponding to the PEPC polypeptide. The band appeared to be more highly expressed in the in_1 plant-controlled interaction than in the 102F26 and 1023 bacterial-controlled interaction. In contrast, in_2 and in_3 plant-controlled tumor-like nodules, which contain few if any bacteroids, lacked the corresponding PEPC polypeptide. These data suggest that expression of enzymically active PEPC is related to both the presence of fixed N_2 and competent bacteroids. This interpretation is supported by the observation that addition of N in the form of NO_3^- and NH_4^+ does not induce GS, GOGAT, and PEPC activity in ineffective nodules containing incompetent bacteroids (8) (SS

Miller, CP Vance, unpublished data).

Previous studies with pea (2) and soybean (11) demonstrated that specific genes are induced for nodule development and function. Induction of these genes is accompanied by transcription of new mRNA species and the translation of new proteins (nodulins) specific for nodules. The mechanism of induction of the genes and the functions of most of their protein products are unknown. Induction of nodule specific genes occurs in both effective and ineffective nodules (2, 10, 11, 23). However, the level of induction is dependent upon host genotype, *Rhizobium* genotype, environment, and effectiveness of the symbiosis (28). Verma *et al.* (28) suggested that the unqiue environment encountered in the nodule may be a factor responsible for the induction of some nodule specific genes. In support of this suggestion, they demonstrated that a group of soybean root proteins which appeared 3 d after infection with *Rhizobium* also appeared after roots were treated with IAA (28). *Rhizobium* bacteria can synthesize IAA and nodule tissue contains more IAA than does root tissue (21, 28).

The increased quantity of PEPC in effective root nodules could be related to the high internal CO_2 concentration of effective nodules. Saturation curves for alfalfa *in vivo* CO_2 fixation (13) showed that nodule internal CO_2 concentration was about 6%, some 20-fold higher than the external environment. This is consistent with the substantially increased *in vitro* activity of PEPC in effective nodules as compared to roots and ineffective nodules.

The immunological cross-reactivity of alfalfa nodule anti-PEPC serum with all of the legume nodules tested demonstrates that the PEPC polypeptide is conserved in the legume species. Further support for conservation of the PEPC polypeptide across species is demonstrated by the cross-reactivity of sorghum leaf anti-PEPC serum with PEPC from *Alnus* nodules (17). The strong immunological cross-reactivity between alfalfa nodule PEPC and soybean nodule PEPC was unexpected because: (a) the contribution of PEPC to nodule N assimilation in alfalfa is strikingly different from that in soybean; (b) alfalfa nodules are indeterminant in growth, while soybean nodules are determinant; and (c) alfalfa PEPC is comprised of two isozymes, while soybean PEPC occurs as five isozymes (6, 19). Alfalfa nodule anti-PEPC serum reacts with both alfalfa isozymes. However, we have not ascertained if all five isozymes that occur in soybean nodules cross-react.

Verma *et al.* (28) have suggested that there are two types of nodulins: (a) C-nodulins which are conserved between many legume species, and (b) S-nodulins that are species specific. Previously documented C-nodulins include leghemoglobin and GS (4, 28). Although PEPC may not fit the criteria for being a nodulin, it is similar to C-nodulins in that it is conserved across many species.

PEPC has been proposed to exist in four major forms, each being associated with a different metabolic pathway yet catalyzing common enzymatic steps (20). The PEPC found in the leaves of C_4 plants functions in the photosynthetic production of malate for carbohydrate synthesis and has been reported to comprise 10 to 15% of the total soluble leaf protein (16). PEPC is present in the leaves of C_3 plant species but in much (5–20 times) lower quantities and functions in the production of malate as a photosynthetic product (16). In the leaves of CAM plants, PEPC functions to produce malate during the dark period which is subsequently used as a CO_2 donor for photosynthesis during the following light period. Finally, PEPC is found in nongreen tissues where its primary role is dependent on the tissue type. In many roots, PEPC functions in malate synthesis which is used for maintenance of ionic balance and perhaps NADPH production. In nodule tissues which export fixed nitrogen in the form of amides, PEPC functions in oxaloacetate production which is subsequently used as a skeleton for amino acid synthesis. The different forms of PEPC can be distinguished both kinetically and chromatographically, yet they appear to share one to many antigenic determinants (3, 14).

Both the recognition of the nodule and root PEPC polypeptides by the maize leaf anti-PEPC serum and the cross-reactivity of the C_4 leaf PEPC with the alfalfa nodule anti-PEPC serum demonstrate conservation of the PEPC enzyme extending across diverse genera. Unfortunately, western blots do not give any information regarding the extent of the differences among enzymes from different sources. However, at least one region of the PEPC polypeptide from these various sources has retained enough identity to be recognized by antibodies prepared against the polypeptide from a distant plant group. The weak reaction of both antisera with the leaf proteins from the C_3 plant species *Triticum* and *Medicago* is likely due to the low levels of PEPC in those plant tissues (16) rather than nonrecognition of the C_3 polypeptide, since other studies have shown that antiserum prepared against the C_4 polypeptide cross-reacts weakly with the PEPC polypeptide from C_3 plants (3, 14). Some weakly staining bands were observed at molecular weights below that of the major PEPC band. These were evident on the blots of the legume nodule proteins and the leaf proteins. Initially, those bands were believed to represent a small degree of nonspecific staining. However, similarly migrating minor bands are present on each of the blots exposed to the two different antisera, indicating that those bands are likely not nonspecific serum binding sites but instead represent breakdown products of PEPC. Although protease inhibitors were included in all extraction buffers, some proteolysis apparently occurred.

In a previous study we reported the subunit mol wt of alfalfa nodule PEPC to be approximately 100,000 (25). The PEPC polypeptide from all sources tested in this study migrated to the same position on an SDS gel as the alfalfa nodule polypeptide, thus indicating another area of PEPC conservation. The active PEPC enzyme from all sources is believed to be a tetramer made up of identical subunits (16). The estimate of 100 kD for the maize polypeptide is in agreement with a previous study, although lower values have been reported (16). The polypeptide mol wt for sorghum has previously been estimated at 90,000 (30).

Others have reported that PEPCs from C_3, C_4, and CAM plant species are immunochemically related (3, 14). Cretin *et al.* (3) immunoprecipitated PEPC from the leaves of several C_3, C_4, and CAM species using sorghum leaf anti-PEPC serum. The antiserum reacted with PEPC from all sources tested but with different efficiencies. Antiserum prepared against the leaf enzyme from the CAM genus *Sedum* was used in Ouchterlony plate assays to test cross-reactivity of the PEPC from species of each photosynthetic type (14). Again, the experiments revealed partial immunologic identity of the leaf PEPCs obtained from different sources. Anti-PEPC serum has also been used to quantitate PEPC in leaf tissue of sorghum (30) and greening leaf tissue of maize (16). The antiserum prepared in this study is the first report of an anti-PEPC serum produced against the enzyme from a nongreen tissue source.

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