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Proline metabolism in N_2-fixing root nodules: Energy transfer and regulation of purine synthesis

[pyrroline-5-carboxylate reductase/proline dehydrogenase (oxidase)/ureides/*Rhizobium*]

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ABSTRACT **N_2-fixing root nodules of soybean (*Glycine max* L. Merr.) convert atmospheric N_2 to ammonia(um) in an energy-intensive enzymatic reaction. These nodules synthesize large quantities of purines because nitrogen fixed by bacteria contained within this tissue is transferred to the shoots in the form of ureides, which are degradation products of purines. In animal systems, it has been proposed that proline biosynthesis by pyrroline-5-carboxylate reductase (P5CR) is used to generate the $NADP^+$ required for the synthesis of the purine precursor ribose 5-phosphate. We have examined the levels, properties, and location of P5CR and proline dehydrogenase (ProDH) in soybean nodules. Nodule P5CR was found in the plant cytosol. Its activity was substantially higher than that reported for other animal and plant tissues and is 4-fold higher than in pea (*Pisum sativum*) nodules (which export amides). The K_m for NADPH was lower by a factor of 25 than the K_m for NADH, while the V_{max} with NADPH was one-third of that with NADH. P5CR activity was diminished by $NADP^+$ but not by proline. These characteristics are consistent with a role for P5CR in supporting nodule purine biosynthesis rather than in producing proline for incorporation into protein. ProDH activity was divided between the bacteroids and plant cytosol, but <2% was in the mitochondria-rich fractions. The specific activity of ProDH in soybean nodule bacteroids was comparable to that in rat liver mitochondria. In addition, we propose that some of the proline synthesized in the plant cytosol by P5CR is catabolized within the bacteroids by ProDH and that this represents a novel mechanism for transferring energy from the plant to its endosymbiont.**

N_2 fixation in legumes is a symbiotic process in which bacteria of the genus *Bradyrhizobium* or *Rhizobium* infect root cells and form specialized organs (nodules) within which N_2 is reduced to NH_4^+. Fixation of N_2 is an energy-intensive process, requiring a total of 25–30 ATP per N atom fixed. Of this total, 12–14 ATP per N are required within the bacteroid to reduce N_2. As much as 10–30% of the total photosynthetic capacity of the plant is used to support this process (1). The energy-yielding metabolite(s) supplied by the host to the bacteroid, the symbiotic form of the bacterium, is not known. However, one attractive suggestion is that bacteroids import and oxidize a nitrogenous compound, such as glutamate (2). The nitrogen fixed in the bacteroid is exported as ammonia(um) to the infected host cell, where it is packaged for export to the rest of the plant. Legumes of temperate origin (e.g., peas) export nitrogen as amides (principally asparagine), whereas legumes of tropical origin (e.g., soybeans) export the ureides, allantoin, and allantoic acid. Ureide biogenesis proceeds by way of synthesis of purine ribonucleotides (3). This pathway is the same as that found in microorganisms, fungi, and animals. The purines so formed are then oxidatively degraded to ureides (3). The estimated peak rate of *de novo* purine biosynthesis necessary to support this flux of ureides from the nodule is 15–20 nmol per hr per mg of nodule fresh weight (4), an extremely high flux even when compared to the elevated rates reported in Ehrlich ascites tumor cells; e.g., 2–8 nmol per hr per mg of cell fresh weight (calculated from the data of ref. 5). The latter rate is 800–4000 times higher than in normal fibroblasts. Thus, ureide biogenesis and export require sustained high rates of *de novo* purine synthesis and the synthesis of essential precursors such as ribose 5-phosphate (Rib5*P*).

Based on analogies with animal systems, purine biosynthesis may be regulated by the levels of Rib5*P* produced via the oxidative limb of the pentose phosphate pathway (PPP) (6). The levels of key enzymes of the oxidative limb of the PPP are higher in tissues with elevated purine biosynthetic activity (for example, sarcomas) than in normal tissues (7). In animals, the carbon flux through the PPP is almost certainly limited by $NADP^+$ availability *in vivo* (8). One possible mechanism for increasing the availability of $NADP^+$ is a reduction in which NADPH is the preferred coenzyme. The conversion of pyrroline-5-carboxylate (P5C) to proline catalyzed by P5C reductase [P5CR, L-proline: $NAD(P)^+$ 5-oxidoreductase, EC 1.5.1.2] is such a reduction.

Substantial direct and indirect evidence exists for a role for P5CR in the regulation of *de novo* purine biosynthesis. In erythrocytes, P5C increased the carbon-atom flux through the oxidative limb of the PPP and the rate of synthesis of Rib5*P*, phosphoribosyl pyrophosphate (5-phosphoribosyl diphosphate, *P*Rib-*PP*), and, in turn, purines (6, 9). A critical control in these studies was the lack of any P5C effect in glucose-6-phosphate dehydrogenase-deficient cells (6). Erythrocytes have considerable P5CR activity, despite having little apparent use for the end product, proline, within the cell. The apparent K_m for NADPH for erythrocyte P5CR was a factor of 20 lower than the apparent K_m for NADH. $NADP^+$ severely diminishes erythrocyte P5CR activity, but NAD^+ does not. Furthermore, erythrocyte P5CR activity is not significantly affected by proline (10). These observations suggest that $NADP^+$ is the physiologically important end product of this reaction in erythrocytes. The proposed role of P5CR as a regulator of purine biosynthesis, along with the

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Abbreviations: P5C, pyrroline-5-carboxylate; P5CR, pyrroline-5-carboxylate reductase; ProDH, proline dehydrogenase; Rib5*P*, ribose 5-phosphate; *P*Rib-*PP*, phosphoribosyl pyrophosphate; PPP, pentose phosphate pathway.

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enormously active purine-synthesizing system of nodules, led us to examine the levels and properties of P5CR in nodules. We report here the observation of very high levels of P5CR activity in the host plant cytoplasm in soybean nodules.

Any cell that has high levels of P5CR activity but does not have high requirements for proline for biosynthetic purposes is left with the significant problem of disposing of the proline. One possible solution, proposed in a series of papers (11–13), is that proline carries hydride ions from the cytoplasm into the mitochondria or possibly from one cell to another. The transfer of hydride ions "provide(s) a mechanism for the intercompartmental . . . transfer of redox potential" (14). The crucial features of this mechanism are P5CR activity in the cytoplasm and proline dehydrogenase [ProDH, L-proline:(acceptor) oxidoreductase, E.C. 1.5.99.8] activity in the other compartment. Given the absence of an obvious biosynthetic sink for proline produced by high activity of P5CR in soybean nodules, we looked for ProDH activity in the same tissue. Bacteroid preparations exhibited ProDH activity comparable to that for rat liver mitochondria and higher than that reported for plant mitochondria. Soybean nodule mitochondrial ProDH activity was, at most, only a small fraction of that observed in the bacteroids. In contrast, previous reports indicate that mitochondria are the primary location of ProDH activity in both plant (15) and animal (16) cells.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* L. Merr., cv. Williams 82) were inoculated with a broth culture of *Bradyrhizobium japonicum* 3I1b 110 (from D. Weber, U.S. Department of Agriculture, Beltsville, MD) and grown in the greenhouse with supplemental light as described (4). Peas (*Pisum sativum* L., cv. Alaska) were inoculated with *Rhizobium leguminosarum* 128C56 (Nitragin, Milwaukee, WI). Growth conditions were as for soybeans.

Preparation of Cell Extracts for Enzyme Assays. P5CR activity was measured in crude extracts (Fig. 1) prepared by grinding the tissue (0.5 g per ml of buffer) in 0.1 M potassium phosphate (pH 6.8) in a ground-glass homogenizer on ice. The homogenate was centrifuged at 12,000 × *g* for 20 min. The supernatant (soluble material, a portion of the mitochondria, and other organelles) was assayed for P5CR activity.

For localization of P5CR activity (Fig. 2), 1.2 g of nodule tissue was gently crushed in a mortar and pestle with 10 ml of grinding buffer {20 mM Tricine [*N*-tris(hydroxymethyl)-methylglycine], pH 8.0/1 mM $MgCl_2$/10 mM KCl/10 mM EDTA plus bovine serum albumin at 10 mg/ml} containing 0.4 M sucrose. The extract was passed through one layer of Miracloth, and 2.5 g of extract (containing ≈0.2 g of tissue) was layered onto a sucrose step gradient (2.3 M, 3 ml; 1.9 M, 4 ml; 1.75 M, 4 ml; 1.7 M, 5 ml; 1.65 M, 5 ml; 1.6 M, 3 ml; 1.55 M, 5 ml; 1.45 M, 3 ml; 1.3 M, 3 ml) in grinding buffer. Gradients were centrifuged in a Beckman SW27 swinging-bucket rotor at 4°C. The rotor was slowly accelerated and the gradients were centrifuged at 113,000 × *g* (max) for 5 hr. The gradient was fractionated (1.2-ml fractions) from the top with an ISCO model 85 gradient fractionator.

For localization of ProDH activity (Fig. 3), 3.1 g of nodule tissue was extracted in 10 ml of the same buffer used for the gradient described above, except that bovine serum albumin was deleted. Extract (3.76 g, containing ≈1 g of tissue) was layered onto a sucrose step gradient (2.3 M, 2 ml; 1.75 M, 4 ml; 1.65 M, 6 ml; 1.6 M, 6 ml; 1.55 M, 4 ml; 1.45 M, 6 ml; 1.3 M, 3 ml; 1.0 M, 3 ml). All sucrose solutions contained 20 mM Tricine (pH 8). Gradients were centrifuged at 113,000 × *g* for 7 hr after slow acceleration.

To definitively separate ProDH activity associated with plastids from that associated with bacteroids (Fig. 4), 7 g of nodule tissue was extracted with 14 ml of 50 mM Tricine, pH 8/0.4 M sucrose and centrifuged at 2000 × *g* for 10 min. A portion of this pellet was set aside. The remainder was extensively washed in 50 mM Tricine (pH 8), osmotically shocked by transfer from 1 M sucrose to 25 mM Tricine (pH 8) free of sucrose in order to disrupt plastids and mitochondria, and repelleted at 12,000 × *g*.

Protein and Enzyme Assays. Protein concentration was measured by a micro Kjeldahl procedure. P5CR activity was measured by the radioisotopic method of Phang *et al.* (17). Fumarase and β-hydroxybutyrate dehydrogenase were assayed according to Farnden and Robertson (18). Prior to assay of β-hydroxybutyrate dehydrogenase, aliquots (300 μl) of each sucrose density gradient fraction were subjected to 15,000 psi (103 MPa) in a French pressure microcell to rupture bacteroids. Triose-phosphate isomerase was measured according to Bergmeyer (19). The ProDH assay was based on the transfer of ^3H from C-5 of proline to the medium (12); incubations were for 20 min at 35°C in a mixture containing 0.1 M Tricine (pH 8), L-[5-^3H]proline (ICN) mixed with 4 mM unlabeled L-proline, and cytochrome *c* (18 μg/ml). Cytochrome oxidase was measured essentially as described by Hanks *et al.* (20).

RESULTS AND DISCUSSION

Activity and Properties of P5CR from Nodules and Other Sources. The rate at which crude extracts of soybean and pea nodules and soybean leaves reduced P5C to proline was measured as a function of protein concentration with NADH as reductant (Fig. 1). The velocities calculated from the slopes were 105.5 ± 9.3, 28.1 ± 2.0, and 5.4 ± 1.2 nmol per min per mg of protein for soybean nodule, pea nodule, and soybean leaf extracts, respectively. Although pea nodules need energy to fix N_2, they do not require extraordinary amounts of purines because their fixed nitrogen is transported as amides. Under the nonsaturating conditions of this experiment, P5CR activity in pea nodules was about one-fourth that found in soybean nodules. P5CR activity in soybean leaves, which do not fix N_2 or have any apparent need for extraordinary quantities of purines, was only 5% of the rate in soybean nodules.

The P5CR activities from a number of plant and animal sources are listed in Table 1. Exact comparisons of all the activities are not possible because assay conditions were not always the same. Nonetheless, it is striking that soybean nodules have considerably greater P5CR activity than any other tissue included in this comparison. Soybean nodule P5CR activity was about 4 times higher than the activity reported for water-stressed barley seedlings, which are

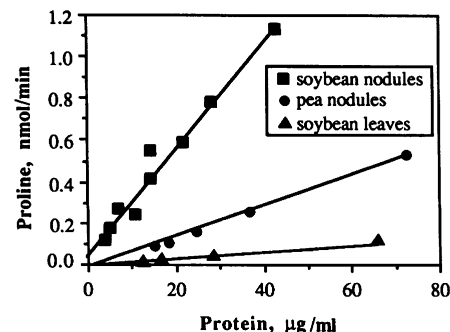

FIG. 1. P5CR activity as a function of protein concentration in extracts of soybean (■) and pea (●) nodules and soybean leaves (▲). Concentrations of P5C and NADH were 0.1 and 0.68 mM, respectively. These concentrations were approximately half the subsequently determined K_m values for soybean nodule extracts. Linear correlation coefficients were 0.966, 0.999, and 0.993 for soybean nodules, pea nodules, and soybean leaves, respectively.

Table 1. Comparison of soybean nodule P5CR activities with activities of P5CR from a variety of plant and animal sources

Tissue	P5C, mM	NADPH, mM	NADH, mM	Velocity	Ref.
Plant					
Soybean nodules	sat.	—	0.68	210 ± 1	
Soybean nodules	sat.	0.68	—	82 ± 5	
Soybean nodules	0.5	—	sat.	281 ± 31	
Soybean nodules	0.5	sat.	—	90 ± 8	
Pea nodules	0.5	—	sat.	47 ± 10	
Pea nodules	0.5	sat.	—	33 ± 3	
Barley seedlings (water-stressed)	sat.	—	0.76	64	21
Pumpkin cotyledons	0.4	—	0.13	10.5	22
Human erythrocytes	sat.	—	sat.	27	10
Rat liver	sat.	—	sat.	12 ± 1	13

Velocity is in nmol per min per mg of protein. Data for soybean and pea nodules are from this study. sat., Saturating.

known to accumulate large quantities of free proline (11 μmol per day per g of fresh weight) to serve as osmoticum (23). In addition, although the specific activity of P5CR from pea nodules was considerably lower than that from soybean nodules, pea nodules still have high levels when compared to most other tissues.

Apparent K_m values for reduction of P5C by soybean nodule extracts were 0.20 ± 0.01 mM (0.68 mM NADH) and 0.12 ± 0.06 mM (0.68 mM NADPH). The 1.7-fold difference in K_m for P5C with NADH vs. NADPH was not statistically significant ($P > 0.1$). The apparent K_m values for NADH and NADPH with P5C concentration held constant at 0.5 mM (about 2.5 and 4 times its apparent K_m value with NADH and NADPH, respectively, as reductant) were 1.55 and 0.06 mM ($P < 0.001$). Some kinetic properties of soybean nodule P5CR are compared to the properties of erythrocyte P5CR and fibroblast P5CR in Table 2. Although the entries in this table are not strictly comparable one to another (since the assay conditions were not all the same), certain important features still emerge. (*i*) For soybean nodule P5CR and erythrocyte P5CR, the apparent K_m for NADPH was 1/25th the apparent K_m for NADH. (*ii*) In contrast, for fibroblast P5CR, apparent K_m values for NADPH and NADH were similar. (*iii*) The influences of $NADP^+$ and proline on soybean nodule and erythrocyte P5CR activities are similar to each other and different from that for fibroblast P5CR. Soybean nodule and erythrocyte P5CR activities were diminished by $NADP^+$ and were insensitive to proline, whereas the reverse was true for the fibroblast enzyme. Thus, the properties of soybean nodule P5CR are strikingly similar to those of the erythrocyte enzyme [for which it was suggested that the enzymatic activity "appears related to NADPH oxidation rather than proline production" (10)] and distinctly different from the properties of the fibroblast enzyme. On the other hand, the properties of fibroblast P5CR (10), particularly the decrease in its activity when proline is present and the absence of any effect of $NADP^+$, are more consistent with a role for this enzyme in the production of proline for incorporation into cell material, primarily collagen, a proline/hydroxyproline-rich protein.

Table 2. Properties of P5CR activity from various sources

Cell type	K_m for NADPH/NADH, mM	K_m for P5C (NADPH/NADH), mM	Relative activity (+ proline/+ $NADP^+$), %
Fibroblast	0.09/0.12	0.20/0.41	12/95
Erythrocyte	0.02/0.39	0.09/0.48	94/33
Soybean nodule	0.06/1.55	0.12/0.20	100/22
Pea nodule	0.48/1.14		

Data for fibroblasts and erythrocytes are from ref. 10. Data for soybean and pea nodules are from this study. Soybean P5CR activities were 100%, 100%, 96%, 96%, and 103% in the presence of 0, 0.5, 2.0, 3.0, and 4.0 mM proline, respectively, with saturating NADH concentration (2.0 mM) as reductant. Soybean P5CR activities were 100%, 58%, 40%, 29%, 22%, and 22% in the presence of 0, 0.5, 1.0, 2.0, 3.0, and 4.0 mM $NADP^+$, respectively. Saturating NADH concentration (2.0 mM) was used as reductant.

Fate of Proline in Nodules. Possible fates of proline produced in nodules include incorporation into protein, export from the nodule via the xylem, accumulation in the nodule, and dehydrogenation. The quantities of proline and hydroxyproline in nodule soluble and cell-wall proteins are insufficient to account for the potential rates of proline synthesis.|| Likewise, the levels of proline in xylem exudate from soybeans are very low (4). Thus, the most plausible fate of proline is its dehydrogenation to P5C catalyzed by ProDH.** ProDH has been found in a variety of prokaryotic and eukaryotic organisms. In both plant (15) and animal (16) tissue, the enzyme has been localized in mitochondria (15).

To determine whether soybean nodules could catalyze the dehydrogenation of proline, we assayed for ProDH activity in crude nodule extracts. The specific activity was comparable to that in rat liver mitochondria (3.3 and 4.3 nmol per min per mg of protein for soybean nodule extracts and rat liver mitochondria, respectively). On a fresh-tissue-weight basis, ProDH activity in soybean nodules (193 μmol per day per g of fresh weight) was about 10-, 500-, and 1000-fold higher than the levels in water-stressed spinach leaves, in wheat shoots, and in corn shoots [17 (ref. 23), 0.4 (ref. 24), and 0.2 (ref. 24) μmol per day per g, respectively]. Since ProDH activity is unstable in many media (25), the measurements reported herein may be an underestimate.

Localization of P5CR and ProDH Activity. By analogy with the location of P5CR in other tissues, we expected to find P5CR activity in the soluble fraction of the soybean nodule extracts. To test this expectation, fractions from a sucrose density gradient were assayed for P5CR activity as well as for the activity of marker enzymes for plastids, mitochondria, and bacteroids. P5CR activity was clearly in the cytoplasmic fraction (Fig. 2).

The subcellular location of the ProDH activity was examined by fractionating nodule extracts on a sucrose density step gradient designed to maximize separation between bacteroids and mitochondria and by differential centrifugation. Activities of marker enzymes (cytochrome oxidase for mitochondria, triose-phosphate isomerase for plastids, β-hydroxybutyrate dehydrogenase for bacteroids) and ProDH were assayed in each fraction (Fig. 3). In previous reports (15, 16), ProDH activity was localized in mitochondria. In this soybean nodule preparation, <2% of the total ProDH activity was in fractions 6–13, which contained virtually all of the activity of the mitochondrial marker enzyme. Approximately 50% of the ProDH activity was found in the bacteroid-containing fractions, 19–21, with the remainder of the activity being at the top of the gradient. The latter corresponded with a peak of triose-phosphate isomerase activity that resulted from plastid breakage. There was also substan-

||About 20% of the amino acid residues in soybean nodules are proline and hydroxyproline (G. Cassab, personal communication). Synthesis of proline for 24 hr at one-half the measured rate of the P5CR reaction would supply all the proline and hydroxyproline present in a mature nodule.

**"Proline dehydrogenase" activity has sometimes been used to designate the $NAD(P)^+$-dependent oxidation of proline by a soluble enzyme at pH > 8.5. However, there is now wide agreement that this activity is associated with P5CR (e.g., see ref. 21). ProDH is a membrane-bound enzyme. In the past, this enzyme has often been referred to as proline oxidase.

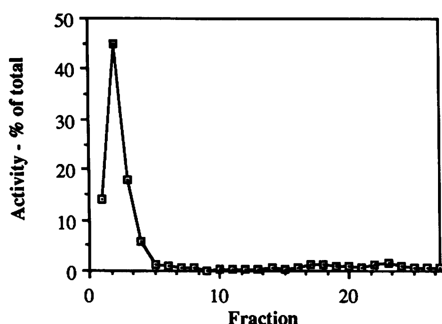

FIG. 2. Localization of P5CR activity in soybean nodule extracts. Extracts were fractionated on a sucrose step gradient. The total P5CR activity in all fractions was 23.8 nmol per min per ml of extract. The top four fractions accounted for 95% of the P5CR activity. Seventy-four percent of the activity of fumarase [a marker for mitochondria and bacteroids (18)] was found in fractions 16–25 (data not shown). Ninety percent of the activity of a marker enzyme specific to bacteroids (β-hydroxybutyrate dehydrogenase) was found in fractions 16–25 (data not shown). There was a peak of triose-phosphate isomerase activity in fractions 1–3 and another between fractions 16 and 18 (data not shown). The first of these is associated with ruptured plastids and the latter with intact plastids. The plastid peak found at the top of the gradient was coincident with the peak of P5CR activity, but there was no P5CR activity in the latter peak. Qualitatively similar results were seen in a replicate experiment.

tial plastid marker-enzyme activity in fractions 19–21, as expected since plastids have a buoyant density quite close to that of bacteroids. The data of Fig. 3 are consistent with ProDH being associated with either bacteroids or plastids. To distinguish between these two possibilities, a crude extract was centrifuged at low speed (2000 × *g*). The pellet contained substantial ProDH, β-hydroxybutyrate dehydrogenase, triose-phosphate isomerase, and cytochrome oxidase activities (data not shown). A portion of the pellet of the crude extract was washed extensively, osmotically shocked to disrupt mitochondria and plastids, and repelleted at 12,000 × *g*. ProDH and β-hydroxybutyrate dehydrogenase activities were essentially the same in the crude and the osmotically shocked preparations, whereas the triose-phosphate isomerase and cytochrome oxidase activities were drastically reduced by the osmotic shock (Fig. 4). These findings demonstrate that ProDH is associated with bacteroids and not plastids or mitochondria. The specific activity of ProDH in washed bacteroids from a similar experiment was 4.9 nmol per min per mg of protein, compared to 4.3 nmol per min per mg in rat liver mitochondria under identical assay conditions. The ProDH activity found at the top of the sucrose density gradient (Fig. 3) awaits further characterization.

Significance of the Results. The novel compartmentation of ProDH within soybean nodules raises the distinct possibility that, together, P5CR and ProDH play an important role in the transfer of redox potential (energy) from the plant cytoplasm to the bacteroids. This hypothesis is presented diagrammatically in Fig. 5. Its central feature is that energy needed by the bacteroids to support N_2 fixation is supplied by the plant as proline. NADPH produced in the host plant cytoplasm is used to reduce P5C to proline. The latter is imported by the bacteroids, where dehydrogenation of proline catalyzed by ProDH is coupled to the bacteroid electron-transport system and oxidative phosphorylation to produce ATP required for N_2 fixation. If proline is oxidized all the way to CO_2 (via P5C, glutamate, oxoglutarate, and the tricarboxylic acid cycle), 35–38 ATP equivalents may be produced (a number sufficient to reduce 2–3 N). If either P5C or glutamate moves from the bacteroid to the host cytosol, then correspondingly fewer ATP equivalents would be produced from the proline carbon skeleton. Fig. 5 also

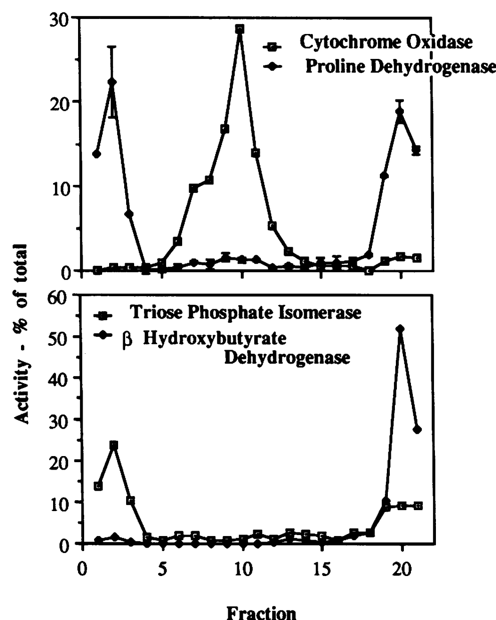

FIG. 3. Localization of soybean nodule ProDH activity. Extracts were fractionated on a sucrose density step gradient, modified to achieve better separation of mitochondria from bacteroids. (*Upper*) ProDH and cytochrome oxidase (mitochondrial marker) activities in each fraction as percent of total activity (56 and 14,640 nmol/min for ProDH and cytochrome oxidase, respectively). Forty-three percent of the ProDH activity was found in fractions 1–3 and 44% in fractions 18–21. Ninety-one percent of the total cytochrome oxidase activity was found in fractions 6–13. Cytochrome oxidase, rather than fumarase, was selected as mitochondrial marker for this gradient, since it consistently exhibited low activity with intact bacteroids. (*Lower*) Triose-phosphate isomerase (plastid marker) and β-hydroxybutyrate dehydrogenase (bacteroid marker) activities; total activities were 38,800 and 350 nmol/ml, respectively. Forty-seven percent of the isomerase activity was in fractions 1–3 and 27% in fractions 18–21. Ninety percent of the β-hydroxybutyrate dehydrogenase activity was in fractions 18–21.

shows N_2 being fixed into NH_4^+, and the NH_4^+ entering the plant host cytosol, where this toxic ion is converted to glutamine and glutamate by the action of glutamine synthe-

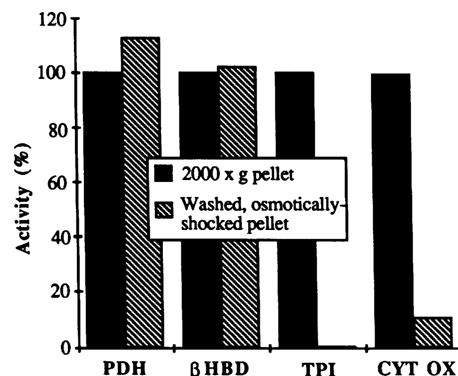

FIG. 4. Comparison of enzymatic activities between a crude soybean nodule bacteroid preparation and a washed, osmotically shocked preparation. Enzymatic activities of ProDH (PDH) and the markers β-hydroxybutyrate dehydrogenase (βHBD), triose-phosphate isomerase (TPI), and cytochrome oxidase (CYT OX) are presented as percent of activity in the crude bacteroid preparation. No significant loss of ProDH or β-hydroxybutyrate dehydrogenase activities occurred as a result of washing and osmotic shock (t = 1.3 for both assays). However, essentially all of the triose-phosphate isomerase activity was lost (t = 17.8; $P < 0.001$), and about 90% of the cytochrome oxidase activity (t = 49; $P < 0.001$) was lost. The triose-phosphate isomerase activity was recovered in the supernatant after centrifugation.

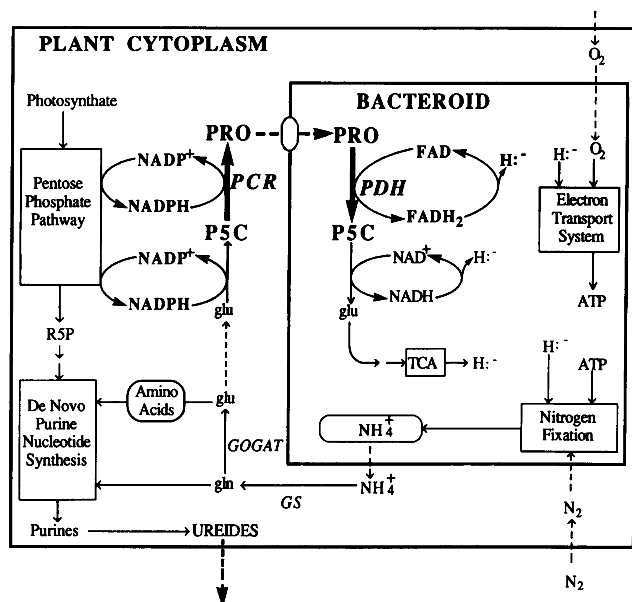

FIG. 5. Diagram of the proposed role of P5CR (PCR) and ProDH (PDH) in N_2-fixing, ureide-exporting nodules. Broken lines represent transport, and solid lines, individual reactions. No attempt is made to represent the known cellular and subcellular compartmentation of these reactions (26). A putative, membrane-bound proline-transport protein is also represented. Reducing equivalents produced in the oxidation of proline, P5C, and glutamate are represented by $H:^-$. Glutamine synthetase and glutamate synthase are represented by GS and GOGAT. See text for discussion. R5P, Rib5*P*; TCA, tricarboxylic acid cycle.

tase and glutamate synthase. While most of the fixed nitrogen from these amino acids is eventually incorporated into ureides, some glutamate might also be available for the synthesis of P5C and, thence, proline. The existence of the necessary enzymatic machinery (high cytosolic "P5C synthase" activity) remains to be determined (see *Note Added in Proof*). Another feature of the hypothesis is that increased flux through the PPP (made possible by the generation of $NADP^+$ in the P5CR reaction) allows the fixed nitrogen to be packaged as ureides for export from the nodule to the plant shoot.[††] Finally, our hypothesis is consistent with the proposal by Kahn *et al.* (2) that energy may be supplied to the bacteroid as a compound that contains nitrogen as well as a carbon skeleton. Proline might also serve as a nitrogen source for bacteroids. Were this the case, the regulation of N_2 fixation, NH_4^+ assimilation, and proline utilization might be intertwined. Indeed, in *Klebsiella* the expression of *glnA* (the structural gene for glutamine synthetase) and the *nif* (N_2-fixation) and *put* (proline-utilization) operons is controlled by the *ntr* (nitrogen-regulatory) system (27). Proline is required for transcriptional activation of the *put* operon. In addition, proline (or P5C or glutamate) might also play a role in the regulation of nitrogen-assimilatory enzymes such as glutamate synthase within the bacteroids.

In summary, on the basis of the levels and location of P5CR and ProDH in soybean nodules, we propose that the activity of these enzymes of proline metabolism results in the transfer of cytoplasmic reducing equivalents from the host plant to its symbiotic partner. This transfer system represents a novel mechanism by which the endophyte receives energy from the host to fuel the process of N_2 fixation. Our results are also consistent with the regulatory role proposed for P5CR in animal cells. $NADP^+$ (produced as a consequence of making the proline needed to satisfy the energy demands of N_2 reduction within the bacteroid) drives the PPP which, in turn, results in a higher rate of synthesis of Rib5*P*, *P*Rib-*PP*, and, ultimately, purine ribonucleotides. This enhanced rate of *de novo* purine ribonucleotide synthesis would allow the N_2 fixed within the nodule to be processed into ureides for export from the nodule to the rest of the plant.

Note Added in Proof. "P5C synthase" activities of 25–45 nmol per min per mg of protein have been observed in five preliminary experiments under nonsaturating conditions.

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[††]We do not mean to suggest that one ureide molecule will be synthesized for every proline produced. A significant fraction of the Rib5*P* may well participate in the nonoxidative limb of the PPP rather than being phosphorylated to *P*Rib-*PP*, the first step of ureide synthesis. This being the case, the ratio of the rate of proline production to the rate of ureide production could be >1. A ratio >1 would be required for proline oxidation in the bacteroid to serve as the sole energy source for fixation of the four N atoms of a ureide molecule. However, bacteroids may well receive and oxidize carbon skeletons other than proline.

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