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Reexamination of the Intracellular Localization of de Novo Purine Synthesis in Cowpea Nodules[1]

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Sucrose and Percoll density gradient centrifugation were used to separate organelles from the central zone tissue of cowpea (*Vigna unguiculata* L. Walp. cv Vita 3: *Bradyrhizobium* strain CB 756) nodules. Enzyme activity analysis has shown that both plastids and mitochondria have a full complement of enzymes for de novo purine synthesis. In vitro activities of individual component enzymes (glycinamide ribonucleotide synthetase, EC 6.3.4.13; glycinamide ribonucleotide transformylase, EC 2.1.2.2; aminoimidazole ribonucleotide synthetase, EC 6.3.3.1; aminoimidazole carboxamide ribonucleotide transformylase, EC 6.3.2.6; and adenylosuccinate-AMP lyase, EC 4.3.2.2) as well as of the whole purine pathway (from ribose-5-phosphate to inosine monophosphate) were similar in the two organelles. No significant cytosolic or bacteroidal activity of any of the purine pathway enzymes was detected on assay. These findings are contrary to earlier studies (M.J. Boland, K.R. Schubert [1983] Arch Biochem Biophys 220: 179–187; B.J. Shelp C.A. Atkins, P.J. Storer, D.T. Canvin [1983] Arch Biochem Biophys 224: 429–441) that concluded that enhanced expression of purine synthesis in nodules of ureide-forming species is localized to plastids. Significantly increased recovery of activity of key pathway enzymes (particularly of labile aminoimidazole ribonucleotide synthetase) coupled with improved assay methods and the use of Percoll in addition to sucrose for gradient centrifugation have together contributed to much higher reaction rates and more definitive analyses of particulate fractions.

Root nodules of many legumes, mainly species in the tribe Phaseoleae, form the ureides allantoin and allantoic acid as the principal organic products of fixed N (Atkins, 1991). The ureides are translocated in xylem to the host and constitute the major source of N for plant growth. Studies based on the use of stable and radioactive isotopes (Atkins et al., 1982, 1988), on the application of specific metabolic inhibitors (Fujihara and Yamaguchi, 1978; Atkins et al., 1980), and on the isolation and characterization of critical enzymes (Atkins, 1991) have established the broad features of the metabolic pathway of ureide synthesis. Ammonia is assimilated in the infected plant cell by Gln synthetase and GOGAT, providing both amide and amino-N to de novo purine synthesis. The nucleotide product of purine synthesis, IMP, is subsequently oxidized through the purine base xanthine to uric acid and the ureides (Atkins, 1991).

In cowpea (*Vigna unguiculata* L. Walp.) and soybean nodules the pathway is highly compartmentalized (Shelp et al., 1983; Schubert, 1986) and involves both infected and uninfected cells of the central tissue zone. Although the initial assimilation of ammonia to form Gln and the oxidation of IMP to xanthine (and subsequently to uric acid) are restricted to the cytosol of the infected cells, GOGAT, along with the complex series of reactions forming the purine nucleotide, has been recovered in subcellular organelles identified as plastids (Boland and Schubert, 1983; Shelp et al., 1983). Evidence for this location is based solely on recovery of organelles from homogenates of nodules following their separation by Suc density gradient centrifugation. More precise methods of localization of individual enzymes of the purine pathway, such as the elegant demonstration of urate oxidase in the enlarged microbodies of uninfected cells using immunogold labeling (Vandenbosch and Newcomb, 1986; Webb and Newcomb, 1987), have not yet been applied.

The rates of purine synthesis demonstrated in cell-free extracts of cowpea nodules are high enough to account for the rates of ureide synthesis occurring during N fixation (Atkins et al., 1982). However, much lower rates of synthesis, typically 10 to 20% for cell fractions and <10% for plastids in the case of cowpea (Shelp et al., 1983), were recovered from isolated infected and uninfected cells and their organelles separated on Suc density gradients (Boland and Schubert, 1982; Shelp et al., 1983). The reasons for such low recovery from organelle fractions have not been investigated, but in view of the fact that the assay for purine synthesis relies on the maintenance of the activity of all 10 enzymes of the pathway from PRPP to IMP plus those required for the synthesis of formyl-THF (Atkins et al.,

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Abbreviations: AICAR, aminoimidazole carboxamide ribonucleotide; AICART, aminoimidazole carboxamide ribonucleotide transformylase; AIRS, aminoimidazole ribonucleotide synthetase; ASAL, adenylosuccinate-AMP lyase; FGAM, formyl glycinamidine ribonucleotide; FGAR, formyl glycinamide ribonucleotide; GAR, glycinamide ribonucleotide; GARS, glycinamide ribonucleotide synthetase; GART, glycinamide ribonucleotide transformylase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; HBD, hydroxybutyrate dehydrogenase; IMPDH, inosine monophosphate dehydrogenase; PRAT, phosphoribosylpyrophosphate amidotransferase; PRPP, phosphoribosylpyrophosphate; SAICAR, succinoamidoimidazole carboxamide ribonucleotide; THF, tetrahydrofolate; TPI, triose phosphate isomerase; XDH, xanthine dehydrogenase.

1982) and the maintenance of a high ATP concentration, it is perhaps not surprising that substantial activity was lost.

Earlier studies on the nature of particulate fractions with purine synthesis activity in cowpea nodules demonstrated the existence of at least two components, based on their different sensitivity to osmotic shock (Atkins et al., 1982). The individual particulate components were not identified, but the data suggest that subsequent density gradient analyses may have overlooked a more complex compartmentalization of the pathway. In the present study both Suc and Percoll have been used as media for density gradients, together with assays for individual purine pathway enzymes, to reinvestigate the intracellular organization of ureide synthesis in legume nodules. Furthermore, assay methods for the complete de novo pathway have been improved so that the activities recovered from density gradient separation of organelles are comparable with in vivo rates of ureide synthesis in nodules.

MATERIALS AND METHODS

Effectively nodulated cowpea (*Vigna unguiculata* L. Walp. cv Vita 3) plants inoculated with *Bradyrhizobium* strain CB 756 were grown in sand culture with a nutrient solution free of combined N under controlled environmental conditions as described previously (Pate et al., 1983). Nodules were harvested from plants 28 to 30 d after sowing and were immediately placed on ice.

Extraction of Nodules

Unless stated otherwise, all operations were carried out at 4°C. Samples of 10 g fresh weight of nodules were gently crushed in a mortar and pestle with 10 mL of a freshly prepared breaking medium containing 0.25 M Suc, 25 mM tetra-sodium pyrophosphate, 2 mM disodium EDTA, 1 mM L-Gln, 0.1 M KCl, 10 mM KH_2PO_4, 15% (w/v) soluble PVP-40, 1% (w/v) BSA (crystalline), 1 mg L^{-1} α_2-macroglobulin (Boehringer Mannheim), and 30 mM ascorbate adjusted to pH 7.5 with KOH. The homogenate was filtered through Miracloth (Calbiochem) and wetted with breaking medium to remove the residual cortical material (Shelp et al., 1983). Any remaining larger material was removed from the filtrate by centrifugation at 200*g* for 2 min. The supernatant served for assay of enzyme activities or was loaded onto density gradients for the separation of organelles.

Extracts for fractionation on Percoll gradients were prepared in the same way but used a different breaking medium. The medium contained 0.4 M sorbitol, 1 mM L-Gln, 5 mM $MgCl_2$, 25 mM KCl, 5 mM KH_2PO_4, 5 mM disodium EDTA, and 1% (w/v) soluble PVP-40 in 25 mM Hepes-KOH (pH 7.2). DTT (5 mM) was also added freshly.

Separation of Organelles on Suc Gradients

For most experiments, 4.5 mL of the supernatant (see above) was carefully loaded onto a 35 to 55% (w/w) linear Suc gradient prepared in 36-mL polyallomer tubes as described previously (Shelp et al., 1983). The gradient contained 50 mM Tes-KOH (pH 7.5), 1 mM L-Gln, 0.1 M KCl, and 0.2% (w/v) BSA. Gradients were centrifuged in a rotor (Beckman SW 28) at 4°C and 24,000 rpm for 25 min. Fractions (1 mL) were collected dropwise from the bottom of the gradient and stored on ice before assay for enzyme activities. These assays commenced approximately 2 h after the nodules were disrupted or, if this was not possible, following storage of fractions at −80°C.

Separation of Organelles on Percoll Gradients

A sample of 4 mL of the nodule extract prepared as outlined above was layered onto 28 g of 60% (w/w) Percoll solution prepared with the breaking medium described above (but omitting PVP-40) and centrifuged at 4°C and 25,000*g* for 60 min in a fixed-angle rotor (Sorvall SS-34). The gradients were fractionated dropwise from below and collected in 1-mL aliquots in tubes containing 20 μL of aqueous 40% (v/v) glycerol:15% (v/v) β-mercaptoethanol solution. High concentrations of Percoll interfered with the assay of some enzymes, so the collected fractions were mixed with an equal volume of breaking medium and centrifuged for 5 min in a microfuge, and the pellet was resuspended in 0.25 mL of the same medium. The "washed" fractions were held on ice for assay of enzyme activities. Assays commenced approximately 2 h after disruption of the nodules or following storage of fractions at −80°C.

In some cases a number of fractions from the Percoll gradients described above were pooled (4–5 mL) and further fractionated on a second Percoll gradient. This gradient was formed from three layers of 40% (2 mL), 30% (3 mL), and 20% (3 mL) (w/w) Percoll (in breaking medium without PVP-40) in 14 × 95-mm polyallomer tubes and centrifuged at 4°C and 4000 rpm for 7.5 min in a rotor (Beckman SW40). The gradients were fractionated into 0.25-mL samples and prepared for enzyme assay as described above for the initial Percoll gradients. Assays commenced approximately 3 h after disruption of the nodules or following storage of fractions at −80°C.

Preparation and Assay of Mitochondria

Mitochondria were prepared from 50 g fresh weight of cowpea nodules using a method based on that of Day et al. (1985). The nodules were thoroughly ground in a chilled mortar and pestle with a total of 150 mL of the breaking medium described above for Suc gradient fractionation. Coarse material was removed from the homogenate by filtering through wetted Miracloth and the filtrate centrifuged at 1,250*g* for 5 min. The supernatant was collected and centrifuged at 11,000*g* for 15 min, and the pelleted organelles were collected and gently resuspended in 5 mL of a wash medium containing 0.6 M Suc, 1 mM L-Gln, 0.1 M KCl, and 0.2% (w/v) BSA in 20 mM Tes-KOH (pH 7.2). The suspension was layered over two 30-mL aliquots of 45% (w/w) Percoll in wash medium and centrifuged for 30 min at 25,000*g* in a fixed-angle rotor (Sorvall SS-34). The bacteroids collected in a broad band toward the bottom of the tube and the plant organelles collected toward the top, but were well resolved from the soluble fraction. The plant

organelle fraction (yield 10–11 mL from the two gradients) was removed and resuspended in 120 mL of wash medium, and, after dispensing to four tubes and centrifuging at 17,500*g* for 15 min, the pellets were collected and combined in 3 mL of wash medium. This was layered on a preformed linear gradient of 0 to 4.4% (w/w) PVP-40 made with 28% (w/w) Percoll in wash medium (30 mL) and centrifuged at 25,000*g* for 45 min as above. The broken organelles collected at the top of the gradient and the intact mitochondria settled in a band toward the bottom. After washing in wash medium the "pure" mitochondria were resuspended in 1 mL and either assayed immediately (approximately 2 h after crushing the nodules) or stored at −80°C.

Cyt *c* oxidase (EC 1.9.3.1) and KCN-resistant alternative oxidase activity were assayed by O_2 uptake using a Clark-type O_2 electrode (Day et al., 1985; Vanlerberghe et al., 1995) to confirm the identity and intactness of mitochondrial preparations.

Enzyme Assays

All assays were carried out at 30°C and, unless stated otherwise, contained 0.1% (w/v) Triton X-100 to ensure complete release of activity from particulate fractions.

GOGAT (EC 2.6.1.53), urate oxidase (EC 1.7.3.3), HBD (EC 1.1.1.30), GDH (EC 1.4.1.3), TPI (EC 5.3.1.1), purine nucleosidase (EC 3.2.2.1), IMPDH (EC 1.2.1.14), and XDH (EC 1.2.1.37) were assayed as described previously (Atkins et al., 1980, 1985, 1989; Shelp et al., 1983). Fumarase (EC 4.2.1.2) was assayed according to the method of Cooper and Beevers (1969).

GARS (EC 6.3.4.13) was assayed by a modification of the method of Schrimsher et al. (1986a). The reaction mixture contained 0.1 M Tricine-KOH (pH 7.8), 12 mM $MgCl_2$, 20 mM Rib-5-P, 290 mM NH_4Cl, 1 mM ATP, 1 mM PEP, 2 units of pyruvate kinase (EC 2.7.1.40; type VII from rabbit muscle, Sigma), enzyme source to be assayed (10–192 μL), and 30 μL of [1-^{14}C]Gly (55 kBq; Amersham) in a final volume of 0.3 mL. The reaction was initiated by the addition of radioactive Gly, and 60-μL aliquots were removed after 0.5, 10, 20, and 30 min and quenched with 9 μL of 30% (w/v) TCA. The ^{14}C-labeled GAR was separated in the void of a NH_4^+-form column (Dowex-50) and equilibrated and eluted with 50 mM ammonium formate (pH 3.3). Radioactivity was measured by scintillation spectrometry with external quench correction.

GART (EC 2.1.2.2) was assayed according to the method of Hall and Atkins (1992) using an analog of 10,formyl-THF, 10,formyl-5,8-dideazafolate (5,8-dideazafolate was a gift from Dr. S.J. Benkovic, Pennsylvania State University, State College), and α,β-GAR (a gift from D.J. Hall, Botany Department, University of Western Australia, Perth).

Assay of AIRS (EC 6.3.3.1) was based on the nonradioactive method of Schrimsher et al. (1986b), which measures the rate of production of diazotizable amines in a colorimetric assay. The reaction mixture contained 0.1 mM β-FGAM, 150 mM KCl, 20 mM $MgCl_2$, and 2 mM ATP in 50 mM Hepes-KOH (pH 7.7). The β-FGAM was synthesized from excess FGAR and FGAM synthetase (extracted and purified from *Escherichia coli* strain Tx635/pJS113, as described by Schendel et al. [1989]; the *E. coli* strain overexpressing FGAM synthetase was a gift from Dr. J. Stubbe, Department of Chemistry, Massachusetts Institute of Technology, Cambridge) according to the method of Schrimsher et al. (1986b). FGAR was synthesized from GAR and 10,formyl-THF using purified GART (purified from cowpea nodules; Hall and Atkins, 1992). 10,Formyl-THF was prepared fresh under anaerobic conditions from folinic acid (5,formyl-THF-calcium salt, Sigma) as described by Stover and Schirch (1990).

ASAL (EC 4.3.2.2) was assayed in a reaction mixture containing 50 mM Tricine-KOH (pH 8.0) and 0.64 mM adenylosuccinate by the change in A_{280}. ASAL also catalyzes cleavage of fumarate from SAICAR (Zalkin and Dixon, 1992), but is more conveniently assayed by the cleavage of adenylosuccinate.

Assay of AICART (EC 6.3.2.6) was based on the method of Mueller and Benkovic (1981) and used 10,formyl-THF as the source of formyl groups. Reaction mixtures contained 0.1 M Tricine-KOH (pH 7.5), 0.3 M KCl, 21 μM 10,formyl-THF, and 0.1 mM AICAR in 0.1 mL final volume. All solutions were flushed with Ar. The rate of change in A_{298} was measured before and after the addition of AICAR.

Gradient fractions were assayed for succinate-dependent O_2 uptake in the same reaction mixture as the breaking medium described above for nodule extracts prepared for Percoll gradients (except that PVP and DTT were not included) using a Clark-type O_2 electrode.

Assay of de Novo Purine Synthesis

The method used previously to assay de novo purine synthesis in cell-free extracts (Atkins et al., 1982) was modified to retain more activity of component pathway enzymes and to improve the nature and supply of substrates. Instead of initiating the sequence of reactions with PRPP, Rib-5-P was used as the starting substrate and the activated "C-1" units were supplied as 10,formyl-THF rather than as the crude, complex mixture of C-1-THF derivatives that were used previously (Atkins et al., 1982). The reaction mixture contained 2 mM [1-^{14}C]Gly (37 kBq), 12 mM Rib-5-P, 10 mM L-Gln, 1 mM L-aspartate, 2.2 mM sodium formate, 10 mM $KHCO_3$, 10 mM ATP, 10 mM $MgCl_2$, 10 mM KCl, 200 mM NH_4Cl, 0.1% (w/v) Triton X-100, 25 units of pyruvate kinase, 10 mM PEP, 20 mM β-mercaptoethanol, 1 μM p^1,p^5-di(adenosine-5′) pentaphosphate, 0.27 mM PMSF (Sigma), and the enzyme source to be assayed (200 μL) in 50 mM Hepes-NaOH (pH 7.5). The reaction was initiated in sealed serum vials under an atmosphere of N_2 by the addition of 200 μL of 0.65 mM 10,formyl-THF to make up a final volume of 760 μL. Reactions were terminated and prepared for HPLC analysis as described previously (Atkins et al., 1982). The 10,formyl-THF was prepared fresh from folinic acid as described above.

Conversion of [^{14}C]Gly to [^{14}C]IMP was linear for up to 100 min (see Fig. 6 for time-course data using a purified mitochondrial preparation). However, in all assays of de novo activity, samples were taken a number of different times after initiating the reaction to ensure that linear rates were recorded.

Separation and Identification of Products of Purine Synthesis

The ^{14}C-labeled products of the de novo purine synthesis assays were prepared and separated by ion-suppression, reverse-phase HPLC as described previously (Atkins et al., 1982). The radioactive peak corresponding to IMP was co-chromatographed with authentic nucleotide and hydrolyzed to hypoxanthine (Atkins et al., 1982). This in turn was re-chromatographed by HPLC (as above) and collected, and its identity was finally confirmed by GC-MS of its tert butyl dimethyl silyl derivative, which was prepared by dissolving the dried samples of material from HPLC or of authentic hypoxanthine in 50 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (Pierce) and 200 μL of dry pyridine in a closed vial under N_2. After sonication the mixture was heated at 120°C for 20 min, and the samples were chromatographed on a capillary column (SGE Australia, Melbourne; BP5, 25 m, 0.25-μm film thickness, 0.22-mm i.d.) in a mass spectrometer (Hewlett-Packard HP5890 GC/5970). For hypoxanthine the M-57 ion was highly characteristic at 307 atomic mass units.

RESULTS

Following centrifugation of nodule extracts on continuous density gradients of Suc, each of the three enzymes of the purine pathway assayed (GART, AIRS, and ASAL) was recovered as a "double peak" in particulate fractions (Fig. 1C), which corresponded with marker enzymes for plastids (GOGAT) and mitochondria (GDH) (Fig. 1A). In contrast, two of the enzymes of purine oxidation (IMPDH and uricase) were recovered solely in the soluble fraction at the top of the gradient (Fig. 1B). The levels of purine pathway enzymes associated with the bacteroids (marked by the activity of HBD, Fig. 1A) were extremely low (Fig. 1C). However, significant activity of all three, as well as of the plastid and mitochondrial marker enzymes, was recovered at the top of the gradients in the soluble fraction (Fig. 1). For the three purine synthesis enzymes, 22 to 26% of the total activity was recovered as soluble enzyme, whereas for the two markers GDH and GOGAT, the soluble activity was 40 and 49%, respectively. GOGAT activity but not GDH was also coincident with the bacteroid marker (Fig. 1A), and, like HBD, indicated two partially resolved peaks of bacteroids.

All of the assays shown in Figure 1 contained detergent (0.1% Triton X-100) to ensure complete breakage of organelles in the particulate fractions. Although simply diluting fractions into the assay reaction mixture without detergent released most of the activity associated with the plastids, this was not the case for mitochondrial enzymes. This is shown in Figure 2 for GART. The same was true for the other purine enzymes, as well as for GDH and fumarase.

Centrifugation of nodule homogenates on self-generated 60% Percoll gradients did not separate the mitochondria and plastids. These occurred as a single peak (Fig. 3B), coincident with both GDH (Fig. 3B) and GOGAT activities (Fig. 3A), and with a peak of succinate-dependent O_2 uptake (Fig. 3A), but were well resolved from the soluble fraction at the top of the gradient (fractions 32–40, Fig. 3C) and from bacteroids marked by HBD and a large peak of O_2 uptake activity (fractions 4–10, Fig. 3A). The activities of GART, GARS, and AICART were also coincident with the plastid and mitochondrial markers, and in each case essentially all of the activity was associated with these plant cell organelles; only a trace could be detected at the top of the gradient as soluble activity and none was detected with the

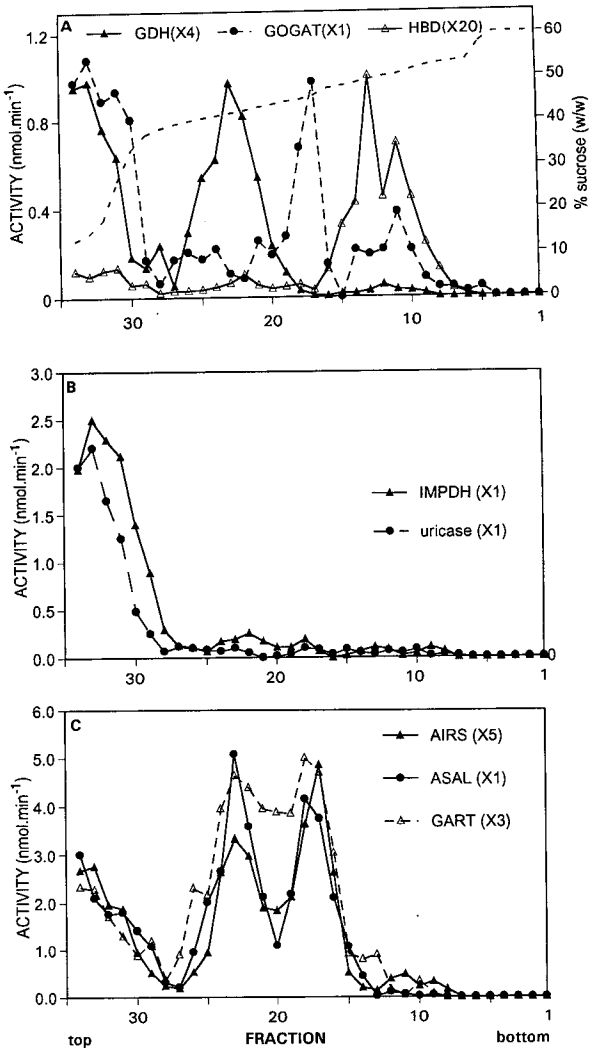

Figure 1. Distribution of enzyme activities in fractions collected after cowpea nodule extract was separated on a 35 to 55% (w/w) linear Suc gradient. After collection Triton X-100 was added to each 1-mL fraction (final concentration 0.1% [w/v]). A, Activity of organelle marker enzymes, GOGAT (●, plastids), HBD (△, bacteroids), and GDH (▲, mitochondria). The dotted line represents the percentage of Suc in the fractions. B, Activity of the enzymes of purine oxidation, IMPDH (▲) and uricase (●). C, Activity of enzymes of de novo purine biosynthesis, AIRS (▲), ASAL (●), and GART (△). Numbers in parentheses indicate the factor by which the rates of activity should be multiplied to give the actual activity. These factors were used so that the activities of all the enzymes could be plotted on the same axis scale.

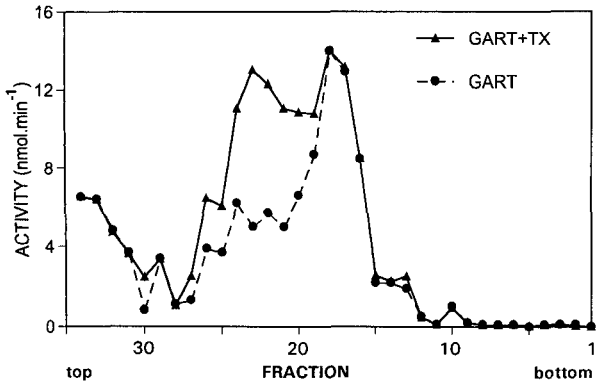

Figure 2. Effect of addition of Triton X-100 (TX) on the activity of GART recovered in assays of fractions collected after cowpea nodule extract was separated on a 35 to 55% (w/w) linear Suc gradient. The activity of GART in each fraction was determined before Triton X-100 addition (●), and then after Triton X-100 was added (▲) to a final concentration of 0.1% (w/v) and the GART assay was repeated.

bacteroids. On the other hand, enzymes of purine oxidation (IMPDH, purine nucleosidase, XDH, and uricase) were not recovered with the plastid/mitochondria peak, but were entirely soluble (Fig. 3C) or, in the case of uricase, were also associated with a dense fraction much lower in the gradient close to the bacteroids (Fig. 3C).

The single peak (fractions 25–30, Fig. 3B) containing activity of mitochondrial and plastid enzymes was collected and further fractionated on a two-step Percoll gradient (Fig. 4). GART activity was resolved into two peaks, one coincident with GDH and fumarase activity and the second, lower in the gradient, with GOGAT and TPI.

Assays of [^{14}C]IMP formation from [1-^{14}C]Gly in fractions separated on a Suc gradient indicated that both mitochondria and plastids contained all of the enzymes necessary for de novo purine synthesis from Rib-5-P and a source of preformed C-1 units (10,formyl-THF) (Fig. 5). The identity of IMP was confirmed by the recovery of [^{14}C]hypoxanthine following acid hydrolysis of the nucleotide and GC-MS analysis of the purine base. HPLC analysis indicated that IMP was the only significant ^{14}C-labeled product of Gly metabolism recovered from the de novo reaction assays; this was also the case for a time-course study of the reaction in which samples were analyzed at various times up to 120 min (Fig. 6). The majority of de novo activity was associated with the plastids and mitochondria (Fig. 5). Less than 30% was recovered in the soluble component at the top of the gradient and negligible activity was found with the bacteroids. The relative activities of the plastid and mitochondrial peaks varied between preparations, but generally the level was slightly lower in the mitochondria.

IMP synthesis by extracts of mitochondria purified by Percoll and Percoll/PVP gradient centrifugation was essentially linear for close to 2 h, when almost all of the Gly supplied was recovered as nucleotide, and achieved rates equivalent to 1 to 2 nmol min^{-1} g^{-1} fresh weight of nodule (Fig. 6).

DISCUSSION

Results with both Suc and Percoll density gradients clearly show that enzymes of the de novo purine synthesis pathway in cowpea nodules are not restricted to plastids as has been thought (Boland and Schubert, 1983; Shelp et al., 1983), but also occur in mitochondria. The significant activities of component enzymes of the pathway and of the overall sequence, which were recovered in the soluble frac-

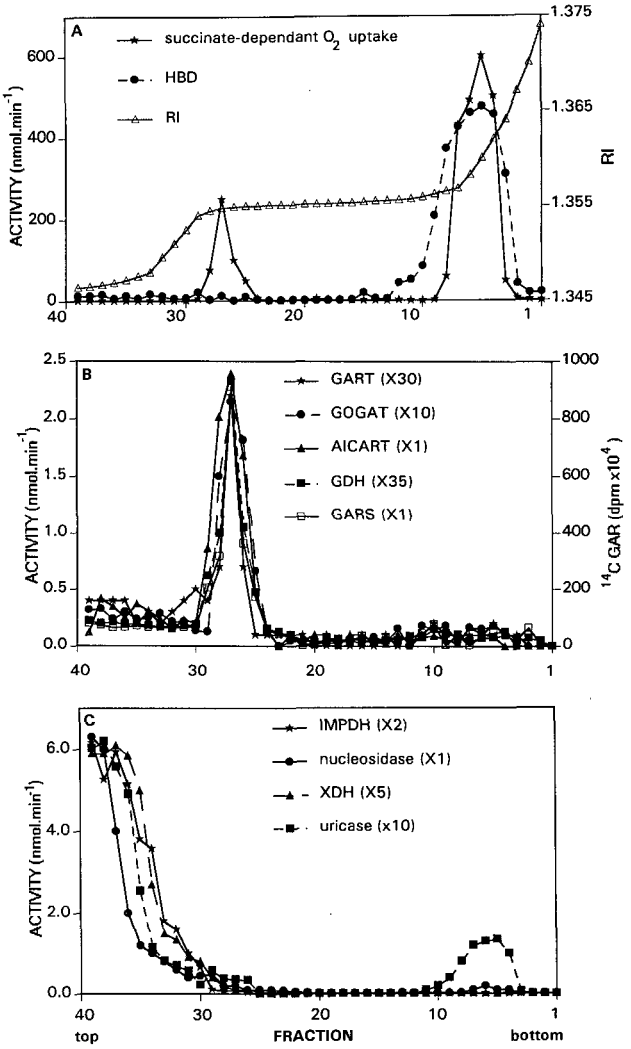

Figure 3. Distribution of organelle marker enzymes and enzymes of purine synthesis and oxidation in fractions collected after cowpea nodule extract was separated in a self-generated 60% Percoll gradient. After collection Triton X-100 was added to each 1-mL fraction (final concentration 0.1% [w/v]). A, HBD activity (●, bacteroids) and succinate-dependent O_2 uptake (★, bacteroids and mitochondria). RI (△) indicates refractive index in each fraction. B, Activity of marker enzymes, GOGAT (●, plastids) and GDH (■, mitochondria), and enzymes of purine synthesis, GART (★), AICART (▲), and GARS (□). C, Activity of enzymes of purine oxidation, IMPDH (★), nucleosidase (●), XDH (▲), and uricase (■). In all cases the numbers in parentheses indicate the factor by which the rates of activity should be multiplied to give the actual activity. These factors were used so that the activities of all the enzymes could be plotted on the same axis scale.

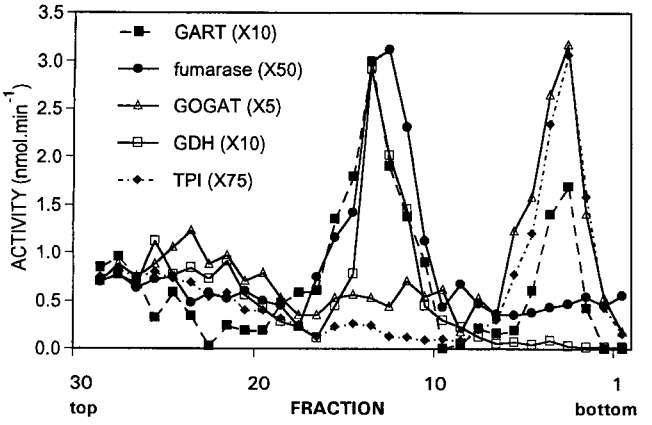

Figure 4. Distribution of marker enzymes and GART in fractions separated in a two-step Percoll gradient (20/30/40%). Fractions containing the "peak" corresponding to mitochondria and plastids collected from a Percoll gradient similar to that shown in Figure 3 (fractions 25–30) were pooled and further fractionated on a two-step Percoll gradient; 0.25-mL fractions were collected and treated with Triton X-100 (final concentration 0.1% [w/v]). Each fraction was assayed for activity of GART (■) and the organelle marker enzymes fumarase (●, mitochondria), GDH (□, mitochondria), GOGAT (△, plastid), and TPI (◆, plastid). Numbers in parentheses indicate the factor by which the rates of activity should be multiplied to give the actual activity. These factors were used so that the activities of all the enzymes could be plotted on the same axis scale.

tion of Suc gradients (23–30%), were probably the result of organelle disruption during centrifugation. There was essentially no soluble activity of purine synthesis enzymes recovered from extracts centrifuged in Percoll gradients, indicating that negligible organelle disruption had occurred. Thus, if there was a cytosolic pathway in nodules in addition to those of the two organelles, its level of expression was extremely low.

The superior performance of Percoll in minimizing organelle breakage is also indicated by the recovery of particulate uricase activity (Fig. 3C), which was not accomplished with the Suc gradients (Fig. 1B). The particulate uricase is likely to be contained in microbodies (Webb and Newcomb, 1987) and, although the majority of the enzyme was solubilized, at least some was retained in these organelles in Percoll.

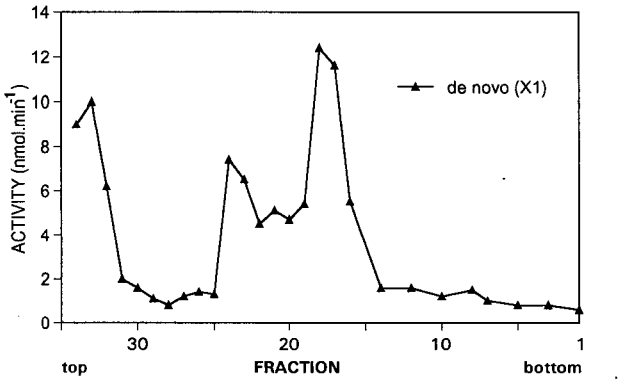

Figure 5. Assays of de novo purine biosynthesis (from Rib-5-P to IMP) in fractions separated by continuous Suc density gradient centrifugation of cowpea nodule extract. The fractions were obtained as described in the legend of Figure 1. The formation of [^{14}C]IMP from [1-^{14}C]Gly was measured in each fraction after the addition of Rib-5-P, Gln, aspartate, 10,formyl-THF, HCO_3^-, and ATP to the reaction mixture containing 0.1% Triton X-100.

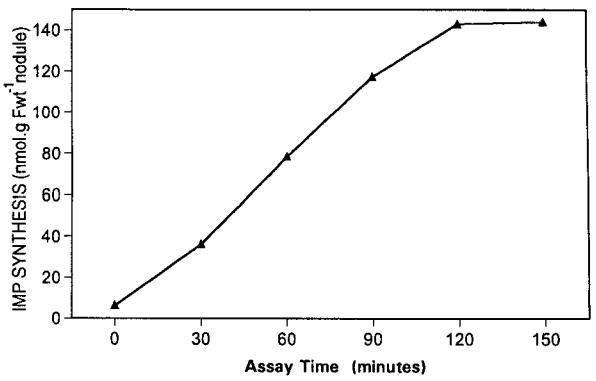

Figure 6. Time course of IMP synthesis (▲) from Gly by an extract of purified mitochondria prepared from cowpea nodules. Mitochondria were purified from nodules using Percoll and Percoll/PVP gradient centrifugation. The formation of [^{14}C]IMP from [1-^{14}C]Gly in a mitochondrial extract was measured after addition of Rib-5-P, Gln, aspartate, 10,formyl-THF, HCO_3^-, and ATP to the reaction mixture containing 0.1% Triton X-100. Formation of IMP was confirmed by HPLC and after hydrolysis to hypoxanthine by GC-MS of its *tert*-butyldimethylsilyl derivative.

The relatively gentle method used for preparations separated by gradient fractionation did not significantly disrupt the cortex of the nodule (Shelp et al., 1983). Thus, the organelles recovered in gradients were derived predominantly from the central tissue zone. Both infected and uninfected cells of this zone contain mitochondria and plastids and both could have contributed organelles to the isolated fractions following centrifugation. However, because plastids in uninfected cells contain very large starch grains (Shelp et al., 1983) and those in infected cells are much smaller, it seems likely that most were recovered from the latter. Similarly, the frequency of mitochondria in infected cells is extremely high (Millar et al., 1994), and, although both cell types occur with equal frequency in the central tissue zone of cowpea nodules, the infected cells are roughly 10 times the volume of uninfected cells (Dakora and Atkins, 1990, 1991). Therefore, most of the mitochondria recovered from gradients were also likely to have come from infected cells. These conclusions are consistent with earlier assays of de novo purine synthesis in separated infected and uninfected cells of cowpea nodules, which showed that the rate in uninfected cells was at most 16% of the activity of the central tissue (Shelp et al., 1983).

There are a number of reasons why the earlier studies failed to detect purine synthesis in mitochondria. Probably the most important was the relatively low level of de novo activity recovered from Suc gradients. Reexamination of the gradient separation data presented by Shelp et al. (1983) does indeed indicate a small "shoulder" of activity on the plastid peak (in figure 3 of Shelp et al., 1983) that could have been due to mitochondria. However, the rates were so low that this was ignored. The activity recovered in

the present study was roughly 10 to 20 times that found previously (approximately 1–2 nmol min^{-1} g^{-1} fresh weight in Fig. 6 compared with 0.1–0.5 nmol min^{-1} g^{-1} fresh weight in Shelp et al. [1983]) and, unlike the earlier assays in which significant ^{14}C accumulated in FGAR and AICAR as well as in IMP (Atkins et al., 1982, 1984), only IMP accumulated in those described here. AIRS is particularly labile, requiring a high level of K^+ ion and Gln in the extraction buffer, as well as in all reaction mixtures, to maintain activity (C.A. Atkins and P.J. Storer, unpublished data). Loss of significant AIRS activity would be expected to cause FGAR to accumulate. Similarly, the accumulation of AICAR noted previously (Atkins et al., 1983) could have been due to a loss of transformylase activity, but might also have resulted from a low concentration of suitable C-1 donor in the reaction mixtures. In the present study a defined source, 10,formyl-THF, was used instead of an undefined mixture of THF derivatives, which would have contained some 10,formyl-THF (Tatum et al., 1977), but which could also have contained inhibitors of the C-1-dependent reactions. It was noted earlier (Atkins et al., 1983) that the high level of ATPase activity in crude extracts and in soluble fractions from gradients can severely limit the rate of purine synthesis assayed. In the present study this potential limitation was minimized by including an effective ATP regeneration system (PEP/pyruvate kinase) as well as an ATPase inhibitor in all de novo assays. As a result linear rates of conversion of Gly to IMP were recorded, essentially until the substrate was exhausted, whereas in earlier studies (Atkins et al., 1982) some assays only remained linear for 15 min due to AMP accumulation. Finally, the de novo assays used earlier relied on precipitation of the enzymes of the pathway with PEG to concentrate the activity for assay (Atkins et al., 1982). The higher levels of activity recovered in this study avoided precipitation and so precluded any differential losses of activity among the component enzymes of the pathway.

Although the levels of individual purine pathway enzymes differed slightly between the two organelles, both appear to have the enzymic potential to contribute significantly to ureide synthesis in cowpea and soybean nodules. However, the purine pathway has a substantial requirement for ATP (7 mol ATP/mol purine), so that the rate at which plastids and mitochondria synthesize IMP in vivo may be quite different. Similarly, the rate of synthesis of other pathway substrates (Gly, aspartate, Gln, and 10,formyl-THF) and/or their rate of entry into the organelles may determine the extent to which each contributes to ureide synthesis. The de novo assays used Rib-5-P as a starting substrate, indicating that both organelles have the capacity for PRPP synthesis (established previously by Le Floc'h and Lafleuriel [1983] for mitochondria from *Helianthus tuberosus* L.). However, in both cases the assays contained a preformed source of C-1 units (10,formyl-THF), so any difference in the nature and capacity for C-1 synthesis in the two organelles would not have been a factor. Previous studies have demonstrated the expression of Ser hydroxymethyltransferase activity in plastids from cowpea (Shelp et al., 1983) and soybean (Mitchell et al., 1986) nodules. Its presence in plant mitochondria has also been well established (Turner et al., 1992; Besson et al., 1995). This seems the likely source of both Gly and C-1 units (as 5,10-methylene THF), but direct evidence for its function in purine synthesis in vivo has not yet been presented.

Although nongreen plastids (proplastids, leucoplasts, and amyloplasts) from a number of tissues have been shown to contain functional glycolytic and oxidative pentose phosphate pathways (Douce et al., 1989), these are unlikely to generate sufficient ATP in situ to support purine synthesis. On the other hand, both mitochondria and amyloplasts of cultured sycamore cells exhibit adenylate carrier proteins, which, in the case of plastids, have been shown to catalyze ATP uptake (Akazawa et al., 1989). Thus, mitochondrial oxidative phosphorylation in the infected cell could well be the major source of ATP for purine synthesis both in mitochondria and in plastids. Furthermore, because O_2 supply limits respiration in the infected cell (Hunt and Layzell, 1993), the extent to which each organelle contributes purines to ureide synthesis may well reflect fluctuations in O_2 supply.

Although purine synthesis in plastids might be dependent on mitochondria for ATP, the pathway in mitochondria would in turn be dependent on plastids as a source of amino-N. GOGAT is located in plastids in both ureide- and amide-forming species (Shelp et al., 1983; Shelp and Atkins, 1984; Schubert, 1986). The substantial levels of soluble activity that are frequently found in Suc gradients (e.g. Fig 1A) are not found in Percoll-based fractionation (e.g. Fig 3B), so they are probably due to more extensive disruption of plastids in high Suc concentrations.

The identification of purine pathway enzymes in both plastids and mitochondria of the same cell (infected cells) raises a number of interesting questions about the nature of the proteins in each organelle, as well as the number and site of their encoding genes. Of the purine pathway enzymes in legume nodules, only one, GART, has been purified sufficiently (Hall and Atkins, 1992) for a comparison to be made with the enzyme from nonsymbiotic tissues or other organisms. The enzyme from cowpea nodules is a monofunctional protein like prokaryotic GART, and lacks the GARS and AIRS activities shown in the trifunctional protein from animals or the bifunctional protein from yeast (Zalkin and Dixon, 1992). When purified, GART from cowpea nodules was separated by SDS-PAGE into two polypeptides (32 and 31 kD). Two similar GART polypeptides (30.5 and 30 kD) were also purified from soybean nodules and cauliflower florets; each showed significant cross-reactivity with polyclonal antibodies raised to the cowpea nodule GART (Hall and Atkins, 1992). Although this does not establish that the nodule enzyme(s) is also present in nonnodule tissues, the fact that two polypeptides were found in each species would be consistent with two GART enzymes in plants, one a plastid isoform and the other a mitochondrial form, both highly expressed in ureide-forming nodules.

cDNAs have been isolated for a number of purine biosynthesis enzymes. These include GARS, GART, and AIRS from Arabidopsis (ATPUR2, ATPUR3, and ATPUR5, re-

spectively; Senecoff and Meagher, 1993; Schnorr et al., 1995), PRAT from Arabidopsis (Ito et al., 1994), soybean (Kim et al., 1995), and mothbean (Kim et al., 1995), and SAICAR from mothbean (Chapmen et al., 1994). All of the full-length clones encode proteins with N-terminal extensions, compared with the *E. coli* enzymes, which are likely to be transit peptides. All but one of these (a PRAT isoform from Arabidopsis) are rich in Ser and are most similar in composition to plastid targeting sequences (von Heijne et al., 1989). In the case of ATPUR5 from Arabidopsis there is also a significant proportion of Arg in the 58-amino acid sequence and Arg is at position −3 compared with the start of the *E. coli* enzyme. Using the program MitoProt (Claros, 1995) a site for presequence cleavage is predicted between amino acids 55 and 56. Thus, although similar to plastid targeting sequences, this sequence also has some similarities with mitochondrial targeting sequences (von Heijne et al., 1989). In vitro targeting experiments will be required to confirm whether the enzymes encoded by particular cDNAs are targeted to plastids, mitochondria, or both.

So far there are few data on whether each purine biosynthesis enzyme is encoded by a single gene or whether there is more than one copy. Southern analysis of ATPUR5 suggests that AIRS from Arabidopsis is encoded by a single gene (Senecoff and Meagher, 1994), but for PRAT there are at least two (ATase1 and ATase2; Ito et al., 1994). Similar data are not yet available for purine genes in any of the ureide-forming legumes. If there is a single gene for AIRS in ureide-forming legumes as there is in Arabidopsis, the question to be answered is how the product of a single gene can be targeted to two different organelles.

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