Enzymes of Ammonia Assimilation in Hyphae and Vesicles of *Frankia* sp. Strain CpI1

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Frankia spp. are filamentous actinomycetes that fix N₂ in culture and in actinorhizal root nodules. In combined nitrogen-depleted aerobic environments, nitrogenase is restricted to thick-walled spherical structures, *Frankia* vesicles, that are formed on short stalks along the vegetative hyphae. The activities of the NH₄⁺-assimilating enzymes (glutamine synthetase [GS], glutamate synthase, glutamate dehydrogenase, and alanine dehydrogenase) were determined in cells grown on NH₄⁺ and N₂ and in vesicles and hyphae from N₂-fixing cultures separated on sucrose gradients. The two frankial GSs, GSI and GSII, were present in vesicles at levels similar to those detected in vegetative hyphae from N₂-fixing cultures as shown by enzyme assay and two-dimensional polyacrylamide gel electrophoresis. Glutamate synthase, glutamate dehydrogenase, and alanine dehydrogenase activities were restricted to the vegetative hyphae. Vesicles apparently lack a complete pathway for assimilating ammonia beyond the glutamine stage.

Filamentous actinomycetes in the genus *Frankia* are N₂-fixing aerobes that form actinorhizal root nodule symbioses with certain nonleguminous plants. In aerobically grown cultures and in most root nodules, N₂ fixation is preceded by the differentiation of nitrogenase-containing structures, the *Frankia* vesicles (20, 25, 32). Vesicles develop at the tips of short hyphal branches and have a thick, multilayered lipid envelope that is proposed to protect nitrogenase from inactivation by O₂ (23, 28, 34, 36). For aerobically cultured *Frankia* spp., the product of N₂ fixation must be transferred in some form to the growing vegetative hyphae. Nitrogen fixation can occur without vesicle formation in microaerobic environments in culture (24) and in nodules of certain actinorhizal plants (38).

One approach to studying the comparative nitrogen metabolism of the vesicles and hyphae is to assay for the presence of enzymes involved in ammonia assimilation; ammonia is the first product of N₂ fixation. Glutamine synthetase (GS; EC 6.3.1.2) is the primary NH₄⁺-assimilating enzyme in most bacteria during NH₄⁺ limitation (37) and in most diazotrophs during N₂ fixation (30). GS uses NH₄⁺ plus glutamate to make glutamine. Glutamine can be used for the biosynthesis of proteins or other metabolites, or with α -ketoglutarate, it can be converted by glutamate synthase (GOGAT; EC 1.4.7.1) to make two glutamates. The glutamate formed is used for amino acid biosynthesis and as a substrate for GS (37).

Glutamate dehydrogenase (GDH; EC 1.4.1.4) and alanine dehydrogenase (ADH; EC 1.4.1.1) have also been reported to participate in ammonia assimilation by the reductive amination of α -ketoglutarate and pyruvate, respectively. GDH has a characteristically high *K_m* for NH₄⁺ and can perform an assimilatory role under conditions of ammonia excess (37). It is the predominant NH₄⁺-assimilating enzyme in NH₄⁺-grown and N₂-fixing cells of certain diazotrophic bacilli (14, 15). ADH can function in assimilation when NH₄⁺ is present in excess in organisms that apparently lack GDH (1, 4, 13, 16).

In previous work on the nitrogen metabolism of *Frankia* sp. strain CpI1, two GSs were characterized (10, 35). GSI is the only form of GS detected in NH₄⁺-grown frankial hyphae. Like many other bacterial GSs, GSI is a dodecamer of identical subunits, each of which can be reversibly adenylylated (10). Cultures grown with a poor nitrogen source like N₂ or glutamate have a highly active, heat-labile GS (GSII) and only low GSI activity (10, 35). GSII is inhibited by glutamine, other amino acids, and a low energy charge (35); no evidence of regulation by adenylylation has been found (10). *Frankia* GSII is similar to the GSII found in members of the family *Rhizobiaceae* in its pattern of induction and in its sensitivity to inhibition by metabolites (8, 9, 11).

The restriction of nitrogenase to the vesicles raises questions about how NH₄⁺ is assimilated during N₂ fixation in *Frankia* spp., since N₂ fixation is spatially separated from the growing hyphal tips of the actinomycete. Addressed in this paper are the activities and distributions of the two frankial GSs, GOGAT, GDH, and ADH in vegetative hyphae and in vesicles of *Frankia* sp. strain CpI1 grown in defined liquid media.

MATERIALS AND METHODS

Organism and culture media. *Frankia* sp. strain CpI1 (*Frankia* catalog no. HFP070101), a *Comptonia peregrina* root nodule isolate (7), was maintained in defined liquid medium with succinate as the carbon source and NH₄Cl as the nitrogen source as previously described (25). To establish N₂-grown cultures, hyphae were collected by centrifugation and suspended in 500 ml of medium without combined nitrogen (25). Cultures were then incubated in cotton-stoppered, 1-liter Erlenmeyer flasks for 4 days at 30°C on a gyratory shaker (New Brunswick Scientific Co., Inc.) set at 150 rpm. NH₄⁺- and N₂-grown cells were collected by vacuum filtration onto filter paper and washed with cold buffer A (10 mM potassium phosphate, pH 6.5) on the filter paper to remove culture medium. The cells were either used immediately for preparing crude extracts or frozen at -80°C.

Vesicle isolation. Vesicles were isolated by a modification of our published procedure (25). Usually, 2 liters of N₂-fixing CpI1 culture was collected on filter paper by vacuum filtra-

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tion, and the mycelia were removed to ice-cold 50-ml centrifuge tubes; cell paste from 1 liter of culture was added to each tube. Cells were suspended in 20 ml of cold 50% (wt/wt) sucrose in buffer A and homogenized on ice with a Polytron tissue homogenizer until microscopic examination revealed that most of the vesicles were detached from the hyphae. Chilled 50% sucrose was added to each tube to a final volume of 30 ml, and the contents were stirred to obtain a uniform suspension of cells in sucrose. After the suspension was overlaid with 5 ml of 10% (vol/vol) glycerol in buffer A, the glycerol-sucrose gradients were centrifuged at $35,000 \times g$ for 1 h at 4°C.

During centrifugation, vesicles banded at the glycerol-sucrose interface. The preparation was essentially free of hyphal contamination, as assessed microscopically. Vesicles were removed from each tube with a Pasteur pipette and transferred to a 17-ml ultracentrifuge tube. They were suspended in buffer A and washed once by ultracentrifugation at $40,000 \times g$ for 15 min at 4°C. Each vesicle pellet was suspended in 0.5 ml of buffer A, transferred to a microcentrifuge tube, and repelleted by centrifugation at $10,000 \times g$ for 10 min at 8°C. This procedure typically yielded 0.5 ml of packed vesicles from 2 liters of culture used. Vesicles either were used immediately or were stored frozen at -80°C.

The pellets from the glycerol-sucrose gradients were composed of vegetative hyphae and some attached vesicles. We enriched for vegetative hyphae by gently disrupting the pellets in buffer A with a Dounce-type tissue homogenizer, diluting the suspension with additional buffer A, and centrifuging at $500 \times g$ for 10 min. This initial centrifugation selectively pelleted the vegetative hyphae, while most of the vesicles and cell debris remained in the supernatant. The absolute degree of purity of the hyphal fraction is difficult to assess in the absence of a quantitative marker; however, vesicles are a minority cell type in the original population and clearly represent less than 1% of the biomass in the hyphal pellet as estimated microscopically. After two washes at $35,000 \times g$ with buffer A to remove the sucrose, the hyphae were used to make extracts immediately or were stored frozen at -80°C.

Cell extracts. Cell extracts were prepared by sonication at 60 W for 20 min with pulsed output on 50% duty cycle in an ice bath. For determining GS activity, extracts were made in 10 mM imidazole-HCl (pH 6.8) with 2 mM MnCl₂ and 1.0% (vol/vol) glycerol. For GOGAT assays, extracts were prepared in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5) with 1 mM dithiothreitol. For ADH and GDH determinations, 10 mM Tris hydrochloride buffer at pH 8.0 with 1 mM dithiothreitol was used to make extracts. Each sonic extract was centrifuged at $35,000 \times g$ for 20 min at 4°C; the supernatant constituted the crude cell extract.

Vesicle extracts were concentrated by (NH₄)₂SO₄ precipitation as follows. While the extract was stirred, solid (NH₄)₂SO₄ was added to 50% saturation; this solution was stirred in an ice bath at 4°C for 15 min. The extract was then centrifuged at $35,000 \times g$ for 20 min at 4°C, and the (NH₄)₂SO₄ pellet was dissolved in GOGAT assay buffer. Previous experiments indicated that (NH₄)₂SO₄ had no adverse effect on GOGAT activity.

Enzyme assays. GS activities were detected by the γ -glutamyltransferase assay (2) at pH 6.8 for GSI and pH 6.5 for GSII as previously described (10). To measure the relative contributions of GSI and GSII to total transferase activity, extracts were heated at 70°C for 7 min, cooled at room temperature for 10 min, and then placed on ice until

assayed for transferase activity. Control samples of each extract were held at room temperature during the heating and cooling procedure. Heating eliminates the GSII contribution to total transferase activity (10).

GOGAT activity was assayed spectrophotometrically by following the rate of NADH or NADPH oxidation (as specified in Results) as the decrease in A_{340} per min at 25°C (5). The 1.0-ml reaction mixtures (final pH, 7.6) contained 1.0 mM α -ketoglutarate, 0.1 mM NAD(P)H, 5.0 mM L-glutamine, 200 mM HEPES, and enzyme. The pH of 7.6 was determined to be optimal for CpII GOGAT in extracts from NH₄⁺-grown and N₂-fixing cells by varying the pH of the assay buffer (200 mM HEPES; data not shown) and measuring the pH after mixing the reaction components. The background oxidation of NAD(P)H was measured for 1 min, and then L-glutamine was added to initiate the specific reaction. GOGAT activity was calculated from the difference in the rates of the decrease of absorbance in the presence and absence of L-glutamine.

The reductive amination reactions of ADH and GDH were measured by monitoring the oxidation of NAD(P)H at 340 nm at 25°C (22) and were initiated by the addition of sodium pyruvate for ADH or sodium α -ketoglutarate for GDH. The 1.5-ml reaction mixtures contained 0.1 mM NAD(P)H, 1.0 mM sodium pyruvate or sodium α -ketoglutarate, 200 mM NH₄Cl, 100 mM Tris hydrochloride (pH 8.6), and enzyme. The difference in the rates of the decrease of absorbance in the presence and in the absence of pyruvate (for ADH) or α -ketoglutarate (for GDH) was used to determine enzyme activity. The pH of 8.6 was determined to be at or near the optimum for both enzymes in preliminary experiments (data not shown).

Gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was done essentially as described by O'Farrell (26). For each gel, the volume of cell extract containing about 10 μ g of protein was electrophoresed. Isoelectric focusing gels (pH 5 to 7) were 10 cm in length by 1.5 mm in diameter. The second-dimension sodium dodecyl sulfate-polyacrylamide gels were 0.7 mm thick with a 2.5-cm, 4% acrylamide stacking gel and a 12.5-cm, 7% acrylamide separating gel with 2.5% bisacrylamide cross-linker. Gels were stained by the modified silver nitrate method (21).

Protein determination. The method of Bradford (6) for protein determination was used with bovine serum albumin as the standard.

Materials. Chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The Polytron tissue homogenizer was from Brinkmann Instruments, Inc., Westbury, N.Y. The low-speed centrifugations were done with a Superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The high-speed centrifugations were done in Ultraclear ultracentrifuge tubes and ultracentrifuge model LB-50B (Beckman Instruments, Inc., Palo Alto, Calif.). Sonications were performed with a Sonifier model 200 (Branson Sonic Power Co., Danbury, Conn.). Isoelectric focusing gels contained LKB ampholines (LKB, Paramus, N.J.).

RESULTS

Activities and distributions of GSI and GSII. Table 1 shows the GS transferase specific activities from a typical heat treatment experiment using extracts from NH₄⁺-grown hyphae, N₂-grown cells (hyphae with attached vesicles), and the two sucrose gradient-derived fractions, the isolated vesicles and the hyphae. The relative contributions of GSI

TABLE 1. Total activity and relative contributions of GSI and GSII in *Frankia* sp. strain Cpl1 cell types

Cell type	GS transferase specific activity ^a in:		% of total activity ^b of:	
	Control	Heated extracts	GSI	GSII
NH ₄ ⁺ grown	1.2	1.2	100	0
N ₂ grown	2.7	0.03	1	99
Gradient pellet	2.2	0.1	5	95
Vesicle	2.4	0.1	4	96

^a Micromoles of γ -glutamyl hydroxamate formed per minute per milligram of protein. All assays were performed in triplicate, and replicates did not vary by more than 5%.

^b Calculated as (activity remaining after heat treatment)/(activity in the unheated control) $\times$ 100.

and GSII in each extract are reported as percentages of the initial activity.

The GS transferase activity of the NH₄⁺-grown cell extract remained unchanged after heating; thus, the heat-labile GSII was not detected in these extracts. All cell extracts from N₂-fixing cultures, including the vesicles, had more than twice the transferase activity of extracts from NH₄⁺-grown cells. In all N₂-grown cell types, GSII accounted for 95 to 99% of the total GS transferase activity.

By coelectrophoresis of purified preparations of GSI and GSII with extracts from the different *Frankia* cell types, the spots on two-dimensional polyacrylamide gels corresponding to GSI and GSII subunits were identified; the relative positions of each protein are illustrated in Fig. 1. GSI subunits appear as two closely appressed spots; the isoelectric point of the adenylylated subunits is slightly more acidic than that of the nonadenylylated form (10). GSI is present on the gels of all extracts derived from NH₄⁺-grown and N₂-fixing cultures (Fig. 1A to D). GSII is present as a heavily stained spot on gels of extracts derived from N₂-fixing cultures (Fig. 1B to D). Another spot, positioned at a slightly higher molecular weight and closely appressed to GSII on these gels, either is an artifact caused by the abundance of GSII in the extracts or is another protein that is coinduced upon nitrogen starvation. We favor the former possibility, since pure preparations of GSII often show two similar spots, depending on the degree of loading, and because the second spot disappears in parallel with GSII when cultures are returned to NH₄⁺-containing medium. The relative staining intensities of the protein spots for each GS on the gels in

TABLE 2. GOGAT and GS specific activities in *Frankia* sp. strain Cpl1 cell types^a

Cell type	NADH-linked GOGAT activity ^b	GS specific activity ^c
NH ₄ ⁺ grown	5	1.3
N ₂ grown	13	13
Gradient pellet	17	13
Vesicle	<0.1 ^d	15

^a Extracts were prepared in 10 mM imidazole-HCl (pH 6.8) with 2 mM MnCl₂, 1 mM dithiothreitol, and 1.0% (vol/vol) glycerol. For GOGAT assays, samples of each extract were dialyzed into 10 mM HEPES (pH 7.5) with 1 mM dithiothreitol. Extracts were assayed with NADH or NADPH. Each value is the average of three replicates; replicates did not vary by more than 5%.

^b Nanomoles of NADH oxidized per minute per milligram of protein.

^c Micromoles of γ -glutamyl hydroxamate formed per minute per milligram of protein.

^d Measurements of the change in A₃₄₀ per minute were less than 0.001, resulting in specific activities of <0.1 nmol of NADH oxidized per min per mg of protein. This value was obtained for all assays with NADPH.

Fig. 1 are roughly proportional to the contributions made by each GS to the total transferase activity reported in Table 1.

Glutamate synthase. To determine if the GS-GOGAT pathway was present in the different frankial cell fractions, extracts were assayed for both enzymes (Table 2). GSII activity was used as a control on the treatment of the extracts during preparation; excessive heating during extraction results in the inactivation of GSII (10) and also of the heat-labile GOGAT (unpublished observation). GOGAT activity was detected only with NADH; no NADPH-linked GOGAT activity was found. The high level of GS activity relative to GOGAT activity is explained by the nature of the assay used for GS; γ -glutamyl transferase activity is an indirect assay for GS and is about 1,000-fold higher than biosynthetic activity as measured by phosphate release from ATP. Thus, total GS and GOGAT activities increase to a similar extent during N limitation.

Hyphae from both NH₄⁺-grown and N₂-grown cultures contained GS and GOGAT activities. The GS specific activity of the NH₄⁺-grown cells was comparable to that presented in Table 1, while the GS specific activities of the N₂-grown cell types were greater. The presence of dithiothreitol in the extraction buffers in this experiment accounted for the increased activities as the result of stabilization of the relatively labile GSII present in these extracts.

The GOGAT specific activities of N₂-grown and hyphal pellet cell extracts were two- to threefold greater than that of the NH₄⁺-grown cell extract. Despite GS activity comparable to that in N₂-grown cells and gradient pellet cells, neither NADH- nor NADPH-linked GOGAT activity was detected in the vesicle extract. To determine if GOGAT was present but at a low level, the preparation was concentrated approximately 10-fold by precipitation with 50% (NH₄)₂SO₄ as described above, but no activity was detected. The possible presence of a GOGAT-inhibiting substance in the vesicle extracts was tested by mixing vesicle extract separately with NH₄⁺-grown cell extract and with N₂-grown cell extract. Following a 1-h incubation in an ice bath, the mixtures were assayed. The resulting activity of each mixture was approximately the same as would be expected for samples diluted with buffer alone (data not shown). Thus, no inhibitor of GOGAT activity was detected in vesicle extracts.

Additional nitrogen metabolic enzymes. ADH and GDH activities were determined for extracts from NH₄⁺-grown, N₂-grown, and vesicle cells (Table 3). Both NADH and NADPH were used to assay these enzymes, but only

FIG. 1. Two-dimensional gels of *Frankia* cell types. The region of the gel containing both GSI and GSII is shown. (A) NH₄⁺-grown hyphae; (B) N₂-fixing culture cells; (C) sucrose gradient pellet-derived cells; (D) isolated vesicles. Top arrows indicate the location of the adenylylated and unadenylylated subunits of GSI (molecular weight, 59,000). Bottom arrows indicate the location of GSII (molecular weight, 43,000). SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE 3. Specific activities of ADH and GDH in *Frankia* sp. strain Cp11 cell types^a

Cell type	NADH-linked ADH activity	NADH-linked GDH activity
NH ₄ ⁺ grown	3.5	3.0
N ₂ grown	4.0	2.0
Vesicles	<0.1 ^b	<0.1

^a Each value (in nanomoles of NADH oxidized per minute per milligram of protein) is the average of three replicates; replicates did not vary by more than 10%.

^b Measurements of the change in A₃₄₀ per minute were less than 0.001, resulting in a specific activity of <0.1 nmol of NADH oxidized per min per mg of protein. This value was obtained for all assays with NADPH.

NADH-linked activity was found. Assays for NADH-linked ADH and GDH in NH₄⁺-grown and N₂-fixing culture cell extracts yielded comparable activities. Neither GDH nor ADH activity was detectable in vesicle cell extracts with the assay conditions used.

DISCUSSION

Frankia actinomycetes produce three cell types: hyphae, vesicles, and spores. Strain Cp11, as grown here, does not sporulate; hence, NH₄⁺-grown Cp11 cultures are a relatively homogeneous suspension of branching hyphae. All frankial isolates, including Cp11, differentiate to form spherical nitrogenase-containing vesicles attached to short stalks along their hyphae when cultured aerobically with N₂ as the sole nitrogen source (33). The purification of vesicles from N₂-grown Cp11 cultures enabled us to study the NH₄⁺ assimilation pathways in vesicles versus the vegetative hyphae. The principle conclusions from this work are that vesicles contain a substantial amount of GSII and a small amount of GSI but do not contain any detectable GOGAT, ADH, or GDH activity.

Frankia sp. strain Cp11 makes two GSs. GSI is found in Cp11 cells growing with NH₄⁺ and glutamine as sole nitrogen sources; GSI levels are greatly diminished in cells growing on glutamate or N₂ (10, 35). GSII is found at high levels in Cp11 cells growing with glutamate or N₂ as nitrogen sources but not in cells grown on NH₄⁺ or glutamine. GSII contributes 95 to 99% of the total GS transferase activity in cells from N₂-grown cultures, including the vesicles. The high GS activity in vesicles is comparable to that in hyphae from N₂-fixing cultures. The observation that vesicles have high GS activity contradicts our initial finding of low GS activity in vesicles (25), reported before *Frankia* sp. strain Cp11 was known to have a second, heat-labile GS (10). The low activity reported for this cell type was most likely the small GSI contribution, with GSII having been inactivated during vesicle isolation or extract preparation.

Hyphae from N₂-fixing cultures have two- to threefold-higher NADH-linked GOGAT activity than do NH₄⁺-grown hyphae. In addition, low, comparable activities of NADH-linked GDH and ADH were found in both NH₄⁺-grown and N₂-grown hyphae. Thus, pathways to form both glutamine (GS) and glutamate (GOGAT and GDH) are present in the hyphae of Cp11. ADH most likely functions only to form alanine, with no role obvious role in NH₄⁺ assimilation, as there was no significant difference in the ADH activities between cells grown with NH₄⁺ and those grown with N₂.

The most intriguing observation is the apparent absence of a complete NH₄⁺-assimilating cycle in the *Frankia* vesicle. Despite GS specific activity comparable to that of the N₂-

grown hyphae, GOGAT, GDH, and ADH were not detectable in vesicle extracts, even when such extracts were concentrated 10-fold. There are several possible explanations for this finding. (i) Enough GOGAT and/or GDH (or even a different GOGAT) may be present in the vesicles to support biosynthetic needs but were not detectable by the methods used; vesicles grow marginally or not at all except under certain defined conditions (29). (ii) Vesicles may produce glutamine for transfer to the hyphae and receive glutamate in return; thus, bulk glutamate formation occurs in the vegetative hyphae. (iii) NH₄⁺ rather than glutamine is transferred from the vesicles to the hyphal cells; if GSII is only partially active in vesicles because of a low energy charge or lack of substrate (glutamate), the assimilation of NH₄⁺ would be blocked. NH₃ may diffuse from the vesicles and be taken up via the hyphal NH₄⁺ permease (17) for assimilation by GS and GOGAT. The diffusion of NH₃ from actively N₂-fixing cyanobacterial heterocysts (18) and from rhizobial bacteroids (3) occurs in other symbioses during N₂ fixation.

Free-living, heterocystous cyanobacteria provide an interesting parallel with *Frankia* spp. During N₂ fixation, the nitrogenase-containing heterocysts and the vegetative cells exhibit differential expression of GS and GOGAT; both cell types contain GS, while GOGAT is restricted to the vegetative cells (19, 27, 31). Thus, glutamine formed by heterocyst GS diffuses into the adjacent vegetative cells, where GOGAT forms two glutamates from glutamine and α-ketoglutarate. The return of one glutamate to the heterocyst completes the nitrogen metabolic loop (12). Similarly, for *Frankia* spp., GS activity is detectable in the nitrogenase-containing vesicles and in the vegetative hyphae, while GOGAT activity is apparently restricted to the vegetative cells.

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