Glutamate and γ-Aminobutyrate Metabolism in Isolated *Rhizobium meliloti* Bacteroids

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Amino acid levels of free-living *Rhizobium meliloti* cells were compared with those of undifferentiated nodule bacteria and mature bacteroids isolated and purified from alfalfa nodules. Glutamate and glutamine levels declined markedly during differentiation while alanine, glycine, and aspartate levels increased in bacteroids. Bacteroids purified anaerobically contained up to 40 nmoles of γ-aminobutyric acid (Gaba) per milligram of protein, while free-living cells lacked this compound. Anaerobiosis caused bacteroid Gaba levels to increase over those observed in bacteroids exposed to oxygen. Comparison of Gaba levels in the cytoplasm of bacteroids isolated anaerobically from several host-microsymbiont combinations showed that the *R. meliloti*-alfalfa symbiosis produced the highest levels of Gaba under these conditions. When succinate, fumarate, malate, or oxaloacetate was incubated with intact bacteroids under argon-oxygen or nitrogen-oxygen mixtures, glutamate levels increased threefold to nearly 30 nmoles per milligram of protein, while Gaba decreased to one fourth of the anaerobic level (40 nmoles/mg of protein). If oxygen was removed from respiring bacteroids by flushing with argon, the changes in glutamate and Gaba were reversed. Studies with the pyridoxal phosphate enzyme inhibitor, amino(oxy)acetate, indicated that Gaba was metabolized by at least two different pathways. One of these pathways involved transamination, and one required succinate plus oxygen and was independent of transamination. Enzymes necessary for the assimilation of ammonia by the glutamate and alanine dehydrogenase systems and for the formation of Gaba and succinic semialdehyde from glutamate had high activities in bacteroids. Transamination from glutamate, alanine, and Gaba was probably responsible for distribution of assimilated nitrogen within the bacteroids. Ammonia resulting from nitrogen fixation was found to be equilibrated between the bacteroids and cytosolic compartments exterior to the bacteroids within the time of bacteroid isolation.

Pathways of ammonia assimilation in legume-*Rhizobium* symbioses have been investigated primarily with the focus on host cell cytoplasmic enzymes operating in root nodules (Dunn and Klucas 1976; Groat and Vance 1981, 1982; Henson *et al.* 1982; Ta *et al.* 1986b, 1988). While the activity of enzymes involved in ammonia assimilatory pathways has been determined in intact nodules and plant cell cytoplasmic extracts and in some bacteroid preparations (Groat and Vance 1981, 1982; Werner and Stripf 1978), purified mature *R. meliloti* Dangeard bacteroids have not been previously examined in detail for these pathways. Glutamate dehydrogenase activity was reported to be very low in *Bradyrhizobium japonicum* Jordan bacteroids (Dunn and Klucas 1976). Glutamine synthetic systems are not essential for symbiotic nitrogen fixation in the *R. meliloti*-alfalfa system (De Bruijn *et al.* 1989).

Mature bacteroids isolated from alfalfa nodules retained coupling between respiration and the reduction of dinitrogen (Miller *et al.* 1988). Only C$_4$-dicarboxylates of the tricarboxylic acid (TCA) cycle were both actively accumulated and able to support nitrogenase activity in the purified bacteroid fraction. Aspartate, while actively accumulated by a relatively low-affinity transport system, did not support this activity (Miller *et al.* 1988; McRae *et al.* 1989b). Glutamate, alanine, L-leucine, other amino acids, and γ-aminobutyrate (Gaba) were neither actively accumulated by nor did they support nitrogenase activity of the isolated bacteroids (McRae *et al.* 1989a, 1989b). ^{14}C-labeled glutamate and ^{15}N-labeled glutamate supplied externally to the bacteroids were reported previously to be substrates for respiration following passive diffusion into the cells (Ta *et al.* 1988). The major products of subsequent metabolism were carbon dioxide and ammonia that accounted for half of the glutamate which entered the cells. Analyses of isolated bacteroids that had been incubated with labeled glutamate showed that the aspartate pool contained about a quarter of the metabolized ^{15}N label, while glutamine and other amino acid pools accounted for the remainder of the metabolized labels. Of the ^{15}N-labeled products formed, only ammonia was found to be exported from the intact bacteroids (Ta *et al.* 1988).

Major pathways of ammonia assimilation operating exterior to the bacteroid periplasmic membrane in alfalfa nodules result in the synthesis of glutamine by the combined action of glutamate synthase (GOGAT, EC 1.4.1.14) and glutamine synthetase (EC 6.3.1.2; Groat and Vance 1981; Ta *et al.* 1988). Asparagine is formed from aspartate and glutamine by asparagine synthetase (EC 6.3.1.1). This compound is translocated within the host and is responsible for the distribution of ammonia exported from bacteroids to host plant cells (Ta *et al.* 1986a). Although Gaba may be exported from alfalfa root nodules (Ta *et al.* 1986b), no evidence exists for equilibration between bacteroid and host cell Gaba pools.

The peribacteroid membrane must be considered as a barrier to free access of host cell cytoplasmic components

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to the mature functioning bacteroids. L-Glutamate is transported by isolated *B. japonicum* bacteroids and supports nitrogenase activity in contrast to *R. meliloti* bacteroids (Miller *et al.* 1988). However, L-glutamate is not transported by the soybean peribacteroid membrane and hence cannot be a major carbon source supporting *in vivo* nitrogen fixation in this system (Udvardi *et al.* 1988). A significant portion (50–60%) but not all of the *R. meliloti* bacteroids used in the present work appear to be enclosed in a peribacteroid membrane (McRae *et al.* 1989b). Lack of passage of the various metabolites considered herein cannot be attributed solely to the relative impermeability of the peribacteroid membrane.

Although compounds other than the TCA cycle dicarboxylates do not support nitrogen fixation in anaerobically isolated *R. meliloti* bacteroids, preliminary studies suggested that glutamate levels within intact bacteroids were dependent on the supply of externally added succinate and oxygen (McRae *et al.* 1989a). Gaba levels were found to be high in isolated bacteroids while glutamine levels were very low. Ambient Gaba concentrations were determined by the previous exposure of the cells to oxygen, suggesting that a complex pathway for assimilation of ammonia was operating within the bacteroids. Glutamate accumulation also has been reported in *B. japonicum* bacteroids (Salminen and Streeter 1987; Streeter and Salminen 1988). The extent of bacteroidal assimilation of ammonia that is necessary to support protein and heme synthesis has been difficult to estimate as have ambient concentrations of ammonia within the bacteroid and host cell cytosols (Streeter 1989). This report considers the metabolism of glutamate and Gaba in *R. meliloti* bacteroids and shows the operation of assimilatory pathways that support amino acid synthesis. Hence, mature bacteroids can replace some amino acid pools without reliance on active accumulation from the external host cell cytoplasm under symbiotic conditions. The possibility that carbon- or nitrogen-bearing compounds are exported from isolated bacteroid cells has also been examined.

MATERIALS AND METHODS

Growth of plants. Five-day-old legume seedlings were inoculated with the appropriate microsymbiont for all symbioses as listed in Table 1. Cultures (1 L) of each indicated strain were grown on a yeast extract-mannitol medium containing 10 g mannitol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgCl_2$, and 0.1 g NaCl. Inoculum cultures were grown to late log phase at 30° C, harvested by centrifugation, and resuspended in sterile distilled water. All bacterial cultures, with the exception of *R. meliloti* strain Balsac (Agriculture Canada Collection), were obtained from the American Type Culture Collection, Rockville, MD, while legume seeds were obtained from the Plant Gene Resources Centre, Agriculture Canada. Plants were grown in 13-cm-diameter pots containing vermiculite, watered with nitrogen-free Hoagland's solution, and maintained under an 18-hr photoperiod for 7 wk as previously described (Miller *et al.* 1988).

Extraction and fractionation of bacteroids. Nodules (8–10 g fresh weight) were harvested at random from the entire legume root system and homogenized in an anaerobic extraction medium containing 180 mM KCl, 50 mM sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES) buffer, pH 7.0, and 0.1% (w/v) bovine serum albumin using the procedure described by Miller *et al.* (1988), except that an argon rather than nitrogen atmosphere was used. The homogenate was then centrifuged at 100 × *g* for 5 min to remove plant debris and recentrifuged at 2,000 × *g* to pellet the crude bacteroid fraction. Separation and purification of the crude bacteroids into bacteria, transforming bacteria, and mature bacteroids were achieved using self-generating Percoll density gradients (McRae *et al.* 1989b). Bacteroids resuspended in 1–2 ml of extraction medium were layered on top of a 55% (w/v) Percoll suspension in the extraction medium and centrifuged at 48,000 × *g* for 30 min. The uppermost band corresponding to the mature bacteroids and the lower band containing nodule bacteria and transformed bacteroid cells were removed, diluted fivefold with 180 mM KCl and 50 mM TES buffer, pH 7.0 (KCl-TES buffer), and washed twice by centrifugation at 5,000 × *g* for 5 min to remove Percoll. The washed cells were layered on top of a 70% (w/v) Percoll suspension in the extraction medium and centrifuged at 48,000 × *g* for 30 min to separate the transforming bacteria (upper band) and the nodule bacteria (lower band). The two bands were collected, and Percoll was removed by washing twice in the KCl-TES buffer. All solutions used

Table 1. Comparison of γ-aminobutyrate (Gaba) and L-glutamate levels in bacteroids isolated anaerobically from several symbioses[a]

Legume	Microsymbiont	L-Glutamate (nmoles · mg⁻¹ protein)	Gaba[b] (nmoles · mg⁻¹ protein)
Alfalfa cultivar Saranac (*Medicago sativa* L.)	*Rhizobium meliloti* Dangeard strain Balsac	10	31
Sweet clover cultivar Denta (*Melilotus alba* Desr.)	*R. meliloti* strain Balsac	9	4
White clover cultivar Rivendel (*Trifolium repens* L.)	*R. leguminosarum* bv. *trifolii* Jordan, ATCC 14480	34	10
Red clover cultivar Arlington (*T. pratense* L.)	*R. l.* bv. *trifolii*, ATCC 14480	41	4
Pea cultivar Little Marvel (*Pisum sativum* L.)	*R. l.* bv. *viceae* Jordan, ATCC 10004	17	1
Bean cultivar Pencil Pod Black Wax (*Phaseolus vulgaris* L.)	*R. l.* bv. *phaseoli* Jordan, ATCC 14482	72	1
Soybean cultivar Harasoy C₃ (*Glycine max* (L.) Merr.)	*Bradyrhizobium japonicum* (Buchanan) Jordan, ATCC 10324	26	3
Lupine cultivar Bitterlupine (*Lupinus albus* L.)	*Bradyrhizobium* sp., ATCC 10319	11	2

[a]Symbiotically effective nodules were obtained from 6-wk-old plants grown on nitrogen-free growth medium as detailed in the text.
[b]Data are mean values obtained from three replicate sets of plants grown in a single experiment under environmentally controlled conditions. SEM did not exceed ± 5% of indicated values.

in the isolation procedure were sealed in vials and purged with argon, and all subsequent manipulations in the extraction process were conducted in an anaerobic glove chamber under argon. Only the mature bacteroid fraction contained significant respiration-linked nitrogenase activity.

Cell lysis. Mature bacteroids suspended in 80% (v/v) ethanol were readily lysed for analysis by sonication for 20 sec with a Branson Sonifier as previously described (McRae et al. 1989c). Sonication in ethanol under these conditions was not sufficient to release amino acids quantitatively from undifferentiated bacteria. It was necessary to suspend these cells in 2 ml of a lysis buffer containing 0.6 M sucrose, 10 mM EDTA, 50 mM TES buffer, pH 8.0, and lysozyme (1 mg/ml). After mixing for 5 min, the solution was diluted to 30 ml with distilled water and then sonicated for 1 min before treatment with 80% ethanol. For comparative purposes, a similar procedure was applied to all the nodule cell types listed in Table 2.

Bacteroid reaction assays. Mature bacteroids resuspended in the KCl-TES buffer, pH 7.0, at approximately 2–3 mg of protein per milliliter were injected (1 ml) into a sealed 37-ml serum vial containing 2 ml of the KCl-TES buffer and a carbon substrate (final concentration, 5 mM). The vials had been previously purged with either nitrogen or argon, and oxygen was added to give 4% (v/v) of the headspace. The reaction was incubated for up to 1 hr in a shaking water bath (120 oscillations per minute) at 20° C (near the bacteroid nitrogenase temperature optimum, Miller et al. 1988), after which the 3-ml reaction assay was quickly removed from the vials and centrifuged for 1–2 min in a microcentrifuge. The supernatant fluid was removed, and the pelleted bacteroids were lysed in 80% ethanol.

Radioactive ^{14}C-labeled substrates. Reaction mixtures contained 5 mM of a ^{14}C-labeled substrate (2,3-^{14}C-succinate, 2-^{14}C-pyruvate, or uniformly labeled α-ketoglutarate or malate), specific activity 0.15 μCi/μmole, dissolved in the KCl-TES buffer, pH 7.0. After incubation for 1 hr under an atmosphere of 4% O_2, 96% N_2, the reaction mixtures were centrifuged at 3,000 × g for 5 min, and the bacteroid cells were washed three times in the KCl-TES buffer to remove any traces of unbound ^{14}C-labeled substrate. Bacteroids were then lysed and centrifuged, and the ethanolic supernatant was reduced to dryness in a rotary evaporator. The residue was dissolved in distilled water, followed by fractionation of the soluble compounds into the basic fraction (amino acids), acid fraction (organic acids), and neutral fraction (sugars) by sequential ion exchange chromatography on Dowex-50 (hydrogen form) and Dowex-1 (acetate form) ion exchange resins. Radioactivity was determined by scintillation counting in an Aquasol-2 cocktail (NEN Research Products, Boston).

Amino acid and ammonia analysis. Sonicated ethanolic extracts from nodule bacteria and bacteroids were centrifuged at 12,000 × g for 20 min. The supernatant was rotary-evaporated to dryness at 35° C and then resuspended in 0.5 ml of 20% (v/v) methanol containing 50 nmoles of α-aminoadipic acid as the internal standard. Aliquots were analyzed for amino acids by high-performance liquid chromatography (HPLC). Following precolumn derivatization with o-phthalaldehyde, amino acids and unknown compounds were separated on a C-18 column with a methanol and water gradient. For ammonia analyses, plant cell supernatant fluids resulting from bacteroid isolation (Miller et al. 1988) or bacteroid cytosol fractions obtained after sonication were rapidly passed through 0.9 × 10 cm columns containing Sephadex G-25 gel filtration medium at 5° C. Small organic molecules and ammonia were eluted with a fixed volume of water. Ammonia was determined after derivatization with o-phthalaldehyde by the method of Corbin (1984). Ammonia levels were calculated on the basis of the protein concentration of the plant cell and bacteroid cytosols.

Enzyme assays. Mature bacteroids from approximately 10 g fresh weight of nodules were resuspended in 8 ml of 10 mM TES buffer, pH 7.0, and sonicated on ice for three 20-sec intervals. Particulate matter was removed by centrifugation at 12,000 × g for 15 min, and the pellet was resuspended in 5 ml of 10 mM TES buffer, pH 7.0. The supernatant fraction containing the cytosolic proteins was applied to a Sephadex G-25 column (2.5 × 25 cm) preequilibrated with 10 mM K_2HPO_4, pH 7.0, and the protein fraction was collected by eluting with 10 mM K_2HPO_4, pH 7.0. All enzyme assays were conducted with aliquots of the cytosolic fraction containing 1–4 mg of protein. Dehydrogenase activity was assayed at 25° C by following changes in absorbance at 340 nm in reaction mixtures containing the following: isocitrate dehydrogenase, 2 mM DL-isocitrate, 0.5 mM $MgCl_2$ and 0.5

Table 2. Amino acid levels of *Rhizobium meliloti* cells at various stages of differentiation in the symbiotic alfalfa system

Nodule cell types	Amino acids (nmoles amino acid · mg^{-1} protein)[a]												
	Asp	Glu	Asn	Ser	Gln	Gly	Thr	Arg	Ala	Tyr	Gaba[b]	Phe	Unknown
Free-living bacteria	0.8	81.8	—[c]	1.6	15.9	4.5	5.3	0.8	4.2	0.8	—	4.9	—
Nodule bacteria	0.3	18.3	0.2	0.9	0.5	3.1	0.2	0.9	2.2	1.5	4.1	1.2	—
Transforming bacteria	3.2	50.0	1.6	4.8	1.7	19.3	3.0	1.2	13.2	4.5	58.0	7.1	+[d]
Mature bacteroids													
Anaerobic[e]	3.9	9.3	1.6	2.2	0.6	10.3	0.9	0.9	10.7	2.6	41.3	0.7	+
Aerobic[e]	3.2	7.0	0.9	1.8	0.5	5.0	1.1	0.8	5.1	2.3	1.4	1.3	+

[a] Data are representative values from a preparation of free-living cells or bacterial cells isolated from 6 g (fresh weight) of 6-wk-old nodules that were grown as given in the text.
[b] γ-aminobutyrate.
[c] Minus (—) indicates unknown not present in amounts comparable to Gaba.
[d] Plus (+) indicates unknown present in amounts comparable to Gaba.
[e] Bacteroids exposed to air or argon during isolation as indicated.

mM NADP$^+$, 20 mM potassium phosphate, pH 7.5; α-ketoglutarate dehydrogenase, 2 mM α-ketoglutarate and 0.5 mM NAD$^+$, 20 mM potassium phosphate, pH 7.5; pyruvate dehydrogenase, 2 mM pyruvate and 0.5 mM each of coenzyme-A, NAD$^+$, cysteine, MgCl$_2$ and thiamine pyrophosphate, TES buffer, 100 mM, pH 7.2; succinic semialdehyde dehydrogenase, 2 mM succinic semialdehyde and 0.5 mM NADP$^+$, potassium phosphate, 100 mM, pH 7.8; alanine dehydrogenase, 2 mM pyruvate, 2 mM NH$_4$Cl and 0.5 mM NADH, TES buffer, 100 mM, pH 8.0; and glutamate dehydrogenase, 2 mM α-ketoglutarate, 2 mM NH$_4$Cl and 0.5 mM NADH, TES buffer, 100 mM, pH 8.0. Malate dehydrogenase, malic enzyme, and fumarase were assayed by the procedures of Stams et al. (1984). Glutamate decarboxylase was assayed at 25° C by the method of Vezina et al. (1989). The reaction product, Gaba, was determined directly by HPLC. Aminotransferase activities were also assayed by direct measurement of reaction products by HPLC after incubation of substrates with aliquots of bacteroid cytosolic protein for 60 min at 25° C. Reaction mixtures contained the following: Gaba, alanine, or sodium glutamate, 1 mM; ketoacid acceptor, 2 mM; and sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES) buffer, 25 mM, pH 7.8. Aconitase activity was estimated by following the increase in ultraviolet absorbance at 280 nm due to cis-aconitate formation. Reaction mixtures contained the following: sodium citrate, 5 mM; cysteine, 1 mM; and potassium phosphate buffer, pH 7.5, 50 mM.

RESULTS

Changes in the cytosolic amino acid profile of each of the *R. meliloti* developmental stages ranging from free-living bacteria to mature bacteroids are outlined in Table
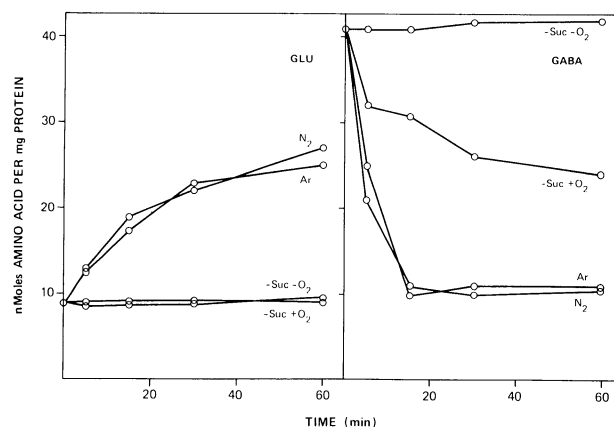

Fig. 1. Effect of succinate on glutamate and γ-aminobutyrate (Gaba) levels in isolated, mature bacteroids. Incubations and analyses were conducted as described in the text. Reactions were started by injection of bacteroids. The left-hand panel shows glutamate (Glu) levels in vials that initially contained 5 mM sodium succinate and were analyzed for L-glutamate at the indicated times. The gas phase contained 4% O$_2$ (v/v) mixed with argon (Ar) or nitrogen (N$_2$) as indicated. Lower curves were obtained when only succinate (Suc) or both succinate and O$_2$ were omitted under either argon or nitrogen. The right-hand panel shows Gaba levels after incubation under the same conditions. Concurrent changes in alanine and aspartate are shown in Figure 3. Results are expressed as mean values from three replicate analyses. Standard errors fell within 10% of the means.

2. The major amino acids in the free-living bacteria were glutamic acid and glutamine at 81.8 and 15.9 nmoles per milligram of protein, respectively, with other free amino acid pools being smaller. No detectable amount of γ-aminobutyric acid (<0.1 nmole of Gaba per milligram of protein) was present in the aerobically grown cells harvested at either mid or late log phase. If the free-living bacteria were washed with bacteroid isolation medium and incubated anaerobically for up to 6 hr under nitrogen alone or with 4% (v/v) oxygen added, no Gaba could be detected. This oxygen level was previously shown to support maximum nitrogenase activity in isolated bacteroids during a 30-min incubation period (Miller et al. 1988). Nodule bacteria showed an amino acid profile similar to free-living bacteria, except that glutamate levels were lower and Gaba was now detectable at 4.1 nmoles per milligram of protein. The transforming bacteria had high levels of Gaba, glutamate, glycine, and alanine at 58, 50, 19.3, and 13.2 nmoles per milligram of protein, respectively. Mature bacteroids had a profile similar to the transforming bacteria with levels of amino acids being slightly lower, especially in the case of glutamate. Very low levels of glutamine (≤ 0.6 nmoles/mg) were observed in bacteroids regardless of the oxygen status of the cells. The composition was substantially altered with regard to several amino acids when the mature bacteroids were isolated aerobically. Glycine, alanine, and especially Gaba were reduced in concentration in these cells. Two unidentified compounds, absent in free-living cells, were detected in the basic fraction of the extracts of mature bacteroids and transforming cells. One unknown peak, equivalent in size to that of Gaba, eluted at a position coincident with α-aminobutyric acid but was not identical to this compound or with known polyamines.

Table 1 compares levels of Gaba and glutamate in the bacteroid fraction from several different legume-microsymbiont combinations. The alfalfa-*R. meliloti* and to a lesser extent the white clover-*R. meliloti* bacteroids contained high levels of Gaba in contrast to bacteroids
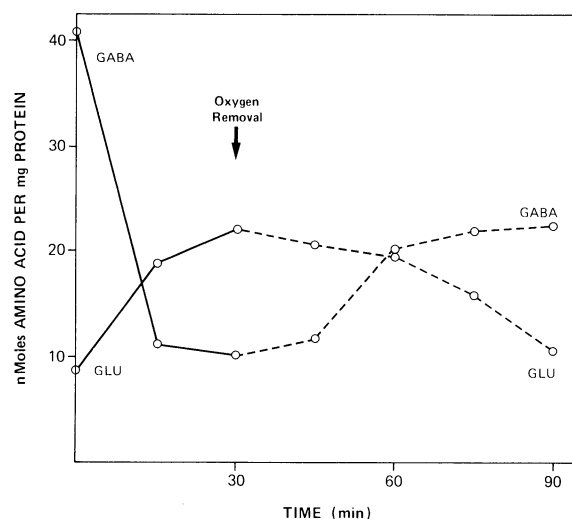

Fig. 2. Effect of oxygen on glutamate (Glu) and γ-aminobutyrate (Gaba) levels in mature bacteroids. Initial 30-min incubation periods were made with succinate under argon plus 4% oxygen. Oxygen was removed from sealed vials at 30 min by flushing with argon. Analyses and SEM values are as given for Figure 1.

isolated from other legume nodules that contained smaller amounts of Gaba. No obvious correlation could be discerned between levels of glutamate and Gaba in the different legume-inoculum systems.

Figure 1 illustrates changes in glutamate and Gaba levels when *R. meliloti* bacteroids isolated anaerobically were incubated for 30 min under conditions that supported maximum nitrogenase activity or under argon (Miller *et al.* 1988). When mature bacteroids were incubated with succinate under either nitrogen or argon containing 4% O_2, glutamate levels rose from 10 to a maximum of 27 nmoles per milligram of protein in 30 min. Glutamate production was independent of nitrogen fixation. Gaba levels dropped from 41 to 10 nmoles per milligram of protein under these conditions. Similar results were obtained on incubation with malate or oxaloacetate (data not shown). The increase in glutamate concentration was dependent on the presence of a TCA cycle dicarboxylate. No decrease in Gaba was observed in the absence of both succinate and oxygen, but Gaba fell to about one half of the original level when oxygen was present in the absence of succinate. Removal of oxygen from the complete reaction mixture headspace after 30 min by flushing reversed the initial rise in glutamate and partially reversed the fall in Gaba as shown in Figure 2. Under these conditions, glutamate levels declined to starting levels while Gaba increased only to about half of the original, giving a final level similar to that observed in Figure 1 when oxygen was added in the absence of succinate.

The concentration of glutamate in bacteroids metabolizing succinate was similar under 4% oxygen plus either argon or nitrogen. If glutamate was synthesized solely from ammonia, the bacteroid pool size of free ammonia would have to be at least 20 nmoles per milligram of protein to account for the increased levels of glutamate observed under argon and oxygen since nitrogen fixation is blocked. Ammonia levels determined for mature bacteroids isolated anaerobically under argon were 29.6 ± 5.0 nmoles of ammonia per milligram of protein, while plant cytosol ammonia levels were slightly lower at 21.6 ± 2.8 nmoles of ammonia per milligram of protein. Bacteroids typically produced 100 nmoles of ammonia per milligram of protein

in 30 min under N_2 gas with 5 mM succinate as the energy source. The presence of dinitrogen did not change glutamate accumulation as shown in Figure 1.

Alanine levels were relatively constant in the presence of succinate and oxygen (Fig. 3). Omission of succinate caused alanine levels to decline by at least 90%, and this process was oxygen-dependent. Aspartate levels dropped significantly when both succinate and oxygen were present. This result suggests that aspartate can participate in further reactions with TCA cycle intermediates or associated metabolites, and indeed, the decreases in aspartate level paralleled the initial, rapid phase of the glutamate decrease but were much smaller than the total increase in glutamate concentration after 30 min (Fig. 1).

Amino(oxy)acetate (AOA) inhibits pyridoxal phosphate-requiring enzymes such as transaminases and glutamate decarboxylase. The effects of this inhibitor on glutamate and Gaba levels are shown in Figure 4. When AOA was added at substrate concentrations (5 mM) to the mature bacteroid suspension and incubated for 30 min under argon plus 4% oxygen (third bar from left axis), glutamate declined but not significantly compared to the zero time control levels. Gaba levels were unaltered under these conditions. The addition of succinate to the reaction mixture caused (second bar from left axis) glutamate to increase by threefold as expected in the absence of AOA. This succinate-dependent increase was amplified up to eightfold in the presence of AOA (fourth bar from left axis). The normal decline in Gaba in the presence of oxygen was completely inhibited by AOA in the absence of succinate (third bar from left axis). If succinate was added with AOA, however, the Gaba levels decreased to the same
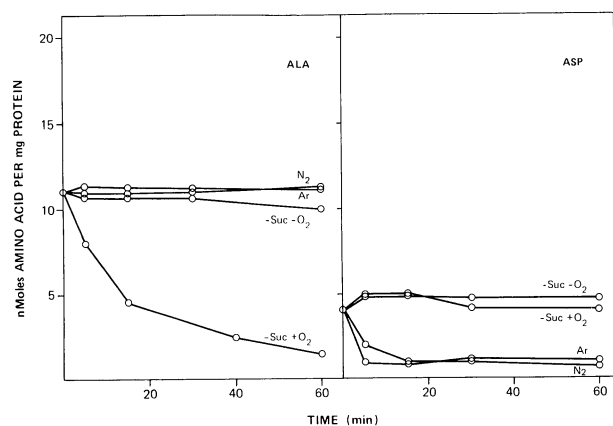
Fig. 4. Effect of 5 mM amino(oxy)acetate (AOA) on glutamate (Glu) and γ-aminobutyrate (Gaba) levels in mature bacteroids. Reaction vials were incubated for 30 min at 20° C under argon plus 4% O_2. Zero time control vessels were equilibrated at 20° C, and the reactions were stopped immediately following the addition of bacteroids by dilution with ethanol as noted in the text. Control vessels (indicated as Suc) contained 5 mM sodium succinate. AOA vessels contained no added substrate unless indicated as Suc + AOA. Glutamate and Gaba were analyzed as stated in the text. Results are expressed as mean values from three replicate vials. SEM values fell within 15% of the means.

Fig. 3. Effect of succinate (Suc) on alanine (Ala) and aspartate (Asp) levels in mature bacteroids. Incubations, analyses, standard errors, and other abbreviations are as given for Figure 1.
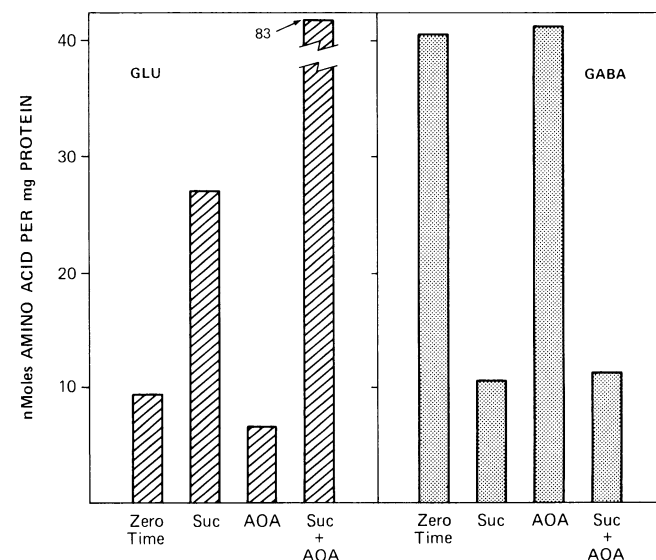
extent as with succinate alone in the absence of AOA.

Pyruvate and α-ketoglutarate are taken up by mature bacteroids at measurable rates (McRae *et al.* 1989b). While neither of these two ketoacids supported high levels of nitrogenase activity, α-ketoglutarate did have noticeable effects on amino acid levels. Incubation of bacteroids with α-ketoglutarate (5 mM) resulted in glutamate levels increasing up to 20.1 nmoles per milligram of protein or about two thirds of the level obtained with succinate. Alanine levels dropped in this experiment in a manner similar to that observed in the absence of succinate (Fig. 3). Pyruvate supplied to mature bacteroids had no effect on glutamate levels, and while alanine levels dropped, this effect was less pronounced than that induced by α-ketoglutarate. Glycine levels (data not shown) and glutamine levels (Table 2) remained constant throughout all treatments, except that adding pyruvate caused the levels of glycine to double in the bacteroids.

The data in Table 3 show that ^{14}C-labeled succinate and uniformly labeled malate were both metabolized in a similar manner under argon or nitrogen plus 4% O_2 with approximately 68–75% of the retained radioactivity accumulating in the basic fraction containing the amino acids. Under similar conditions 85–90% of the radioactivity retained in the cells after feeding 2-^{14}C-pyruvate or uniformly labeled α-ketoglutarate was present in the basic fraction.

A survey of various enzyme activities (Table 4) associated with the mature bacteroids showed the presence of TCA cycle enzymes, including malate dehydrogenase, as well as malic enzyme and pyruvate dehydrogenase. Measured malate dehydrogenase activity was low compared to that reported by Karr *et al.* (1984) for *B. japonicum* bacteroids, indicating that the enzyme may be less stable in *R. meliloti* bacteroids. Isocitrate dehydrogenase activity was also notably low in bacteroid extracts, especially when compared to the high activities reported for the host cell cytoplasmic enzyme (Henson *et al.* 1986) and to the activity found in *B. japonicum* bacteroids (Karr *et al.* 1984). In the present work, no effort was made to stabilize or optimize the measured activity of these enzymes beyond the usual precautions for control of temperature and ionic environments.

Bacteroid metabolism functioned independently of externally supplied pyruvate or glutamate as shown in Figure 5. The two potential ammonia-assimilating enzymes, alanine dehydrogenase and glutamate dehydrogenase, had high activities in the mature bacteroids suggesting that

significant ammonia incorporation into amino acids could occur by these pathways. Glutamate decarboxylase and Gaba-ketoacid aminotransferase activities were found to be lower than the dehydrogenase activities in bacteroid extracts. Two other transaminase systems, alanine-α-ketoglutarate and glutamate-oxaloacetate aminotransferase, were found to have nearly 10 times as much activity as the Gaba transaminases in the extracts. Succinic semialdehyde dehydrogenase was very active in bacteroid extracts and thus removed the potentially inhibitory semialdehyde product of Gaba transamination reactions. Metabolic pathways required for alanine and Gaba synthesis and further metabolism by transamination as shown in Figure 5 were present and presumably functional in mature bacteroid extracts.

DISCUSSION

Microsymbiont cells of the alfalfa-*R. meliloti* symbiosis undergo an extensive process of differentiation subsequent to infection of the host. Since developing alfalfa nodules contain at least three distinct cell types derived from the infecting bacteria (McRae *et al.* 1989c), comparisons of amino acid levels in the isolated cells are indicative of the metabolic consequences of differentiation. Major changes in the carbon and nitrogen metabolism of mature bacteroids allow the altered cells to support the reduction of dinitrogen, specifically by metabolizing succinate, malate, and fumarate or other TCA cycle intermediates to generate the ATP and reducing equivalents required for the nitrogenase reaction. Succinate dehydrogenase mutants are Fix⁻ in phenotype as are mutants unable to transport succinate (Gardiol *et al.* 1987; Engelke *et al.* 1987; Watson *et al.* 1988). Although isolated bacteroids also respire

Table 4. Enzyme activity of mature bacteroid cytosol[a]

Enzyme	Number	Activity (nmoles·h⁻¹·mg⁻¹ protein)
Aconitase	EC 4.2.1.3	1,870
Isocitrate DH[b] (NADP⁺)	EC 1.1.1.42	12,000
Ketoglutarate DH (NAD⁺)	EC 1.2.4.2	3,000
Fumarase	EC 4.2.1.2	370
Malate DH (NAD⁺)	EC 1.1.1.37	190
Malic enzyme (NAD⁺)	EC 1.1.1.38	1,010
Pyruvate DH (NAD⁺)	EC 1.2.2.2	2,610
Alanine DH (NADH)	EC 1.4.1.1	1,240
Glutamate DH (NADH)	EC 1.4.1.2	1,040
Glutamate decarboxylase	EC 4.1.1.15	82
Transaminases		
Gaba[c]: α-ketoglutarate	EC 2.6.1.19	95
Gaba: glyoxylate		84
Gaba: pyruvate		27
Alanine: α-ketoglutarate	EC 2.6.1.2	714
Glutamate: oxaloacetate	EC 2.6.1.1	768
Succinic semialdehyde DH (NADP⁺)	EC 1.2.1.16	2,020

[a] Mature bacteroids were isolated anaerobically from 6-wk-old alfalfa nodules as detailed in the text. Assay conditions are also given in the text.

[b] DH, dehydrogenase. Electron donor or acceptor used in assay is indicated by parentheses.

[c] Gaba, γ-aminobutyrate.

Table 3. Conversion of ^{14}C-carbon substrates to bacteroid metabolites[a]

| Substrate added | Radioactivity of cytoplasmic fractions (% of total retained) | | |
	Basic	Acidic	Neutral
2,3-^{14}C-Succinate	73[b]	27[b]	1[b]
2-^{14}C-Pyruvate	86	9	5
[14]C(U)-Malate	68	30	2
[14]C(U)-α-Ketoglutarate	87	9	4

[a] Incubation, extraction of bacteroid cytoplasmic components, and determination of radioactivity were conducted as described in the text.

[b] Radioactive ^{14}C label retained in the cells after 1 hr, incubation under 4% (v/v) O_2. Mean values of three separate experiments. SEM ≤ 2%. Other values are representative of a single experiment.

endogenous substrates, the addition of a TCA cycle dicarboxylate is necessary for nitrogenase activity (Miller *et al.* 1988).

Several notable differences can be discerned when comparing the properties of *R. meliloti* bacteroids with other species. Not only are Gaba levels uniquely high under anaerobic conditions, but glutamate dehydrogenase activity is also very high. Polyhydroxybutyrate inclusions are not seen in electron micrographs of alfalfa nodules or isolated bacteroids (McRae *et al.* 1989c), whereas such granules are prevalent in free-living *R. meliloti* cells grown on sugars and in *B. japonicum* bacteroids *in situ*.

During isolation, bacteroids were routinely maintained for a short time (30 min) anaerobically (which modifies amino acid levels, Table 1) and without respiratory substrates. Thus, levels of metabolites in the isolated bacteroids may vary considerably from those present in the intact nodules. It seems reasonable to assume that the changes taking place on transfer of the isolated bacteroids to succinate and (low) oxygen media represent a return to conditions nearer to those in intact nodules. During this process glutamate levels rose, and this synthesis was dependent on both succinate (supplying carbon skeletons) and oxygen and was probably catalyzed mainly by glutamate dehydrogenase. Glutamine levels did not vary with the oxygen status of the isolated bacteroids, and hence, glutamine is not the precursor of glutamate. A much greater accumulation of glutamate in the presence of the inhibitor amino(oxy)acetate suggests that a considerable proportion of the glutamate formed normally participates in transamination reactions, presumably providing amino groups for synthesis of other amino acids. *R. meliloti*
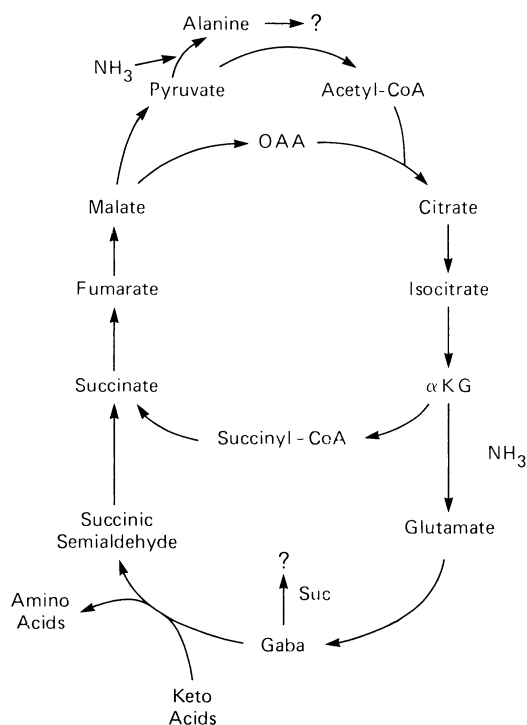
mutants defective in glutamate decarboxylase have been reported to possess low nitrogenase activity (Fitzmaurice and O'Gara 1988). Hence, the Gaba shunt pathway including initial glutamate buildup may be required for full expression of the Fix$^+$ phenotype. Alanine dehydrogenase and β-hydroxybutyrate dehydrogenase were previously reported to increase 10-fold in specific activity during bacteroid differentiation (McRae *et al.* 1989c).

Glutamine levels of anaerobically isolated mature *R. meliloti* bacteroids are very low, while Gaba levels are higher than those observed with isolated bacteroids of other *Rhizobium* species. The presence of oxygen in isolation media caused no significant change in glutamine levels but led to a very marked decline in Gaba. Differences observed between amino acid content of aerobic and anaerobic bacteroids indicate that ammonia production under microaerobic conditions has a pronounced effect on the metabolism of the nitrogen-fixing cells. While much of the ammonia produced rapidly diffuses out of the bacteroids, some must be assimilated to maintain protein, heme, and other biosynthetic pathways essential for maintenance of bacteroid function. The extent of intrabacteroidal ammonia assimilation by anaerobically isolated bacteroids calculated from measured bacteroid nitrogenase activity data was in the range of 15–20% of total ammonia formed in a 30-min incubation period at 20° C. This process cannot be extrapolated beyond the incubation period, however.

Determinations made on root nodule extracts within 10 min of the separation of bacteroids from the plant cell cytoplasmic fraction indicated that, on a protein basis, bacteroid and plant cytoplasms contain nearly equivalent ammonia concentrations. This concentration, between 20 and 30 nmoles of ammonia per milligram of protein, and high glutamate dehydrogenase activities are sufficient to account for the observed accumulation of glutamate within
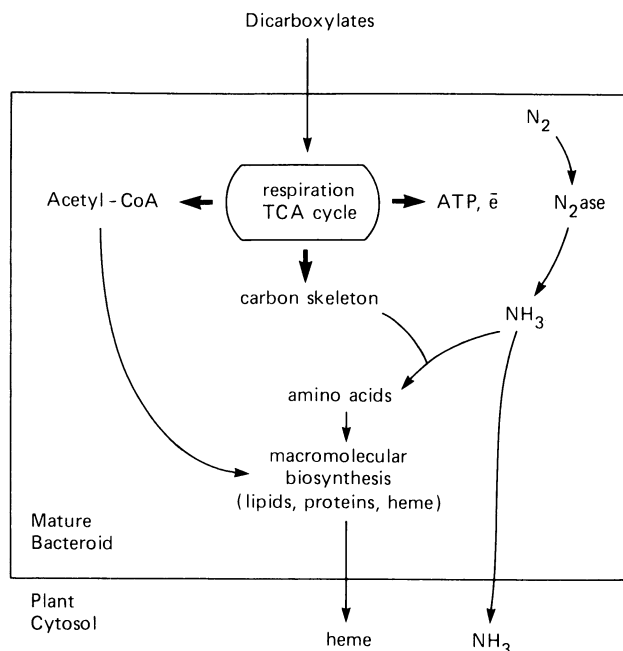

Fig. 5. Tricarboxylic acid, malic enzyme, and γ-aminobutyrate (Gaba) shunt pathways of carbon and nitrogen metabolism in isolated, mature *Rhizobium meliloti* bacteroids. Suc, succinate; αKG, α-ketoglutarate; and OAA, oxaloacetate.

Fig. 6. Overall scheme showing C$_4$-dicarboxylate metabolism and ammonia assimilation within *Rhizobium meliloti* bacteroids. (TCA, tricarboxylic acid.)

bacteroids fed succinate under argon-oxygen mixtures where no ammonia production is possible. However, the rapid decline in aspartate occurring during the first 10 min of glutamate buildup suggests that aspartate may provide some of these amino groups through transamination with α-ketoglutarate. Transamination from Gaba is also a potential source of glutamate nitrogen. The correspondence between declines in Gaba and increases in glutamate concentrations was not 1:1, since Gaba declined even in the absence of transamination (Fig. 3) or succinate oxidation (Fig. 1). Glutamate increased twice as much in the absence of transamination reactions, while Gaba declined to the same extent as was observed in the absence of transaminase inhibition. The extent of continuous internal assimilation of ammonia cannot be assessed from the initial rates of glutamate accretion reported here, since changes in Gaba and glutamate cease before nitrogenase activity declines, as determined in mature bacteroids (Miller et al. 1988).

High levels of Gaba in freshly isolated bacteroids were associated with anaerobic conditions (Table 1) as has been observed in very different systems such as plant shoot cells (Streeter and Thompson 1972). The decrease in Gaba on incubation is dependent on the presence of oxygen, although the decline is accelerated by the addition of succinate (Fig. 1). It is probable that the formation of Gaba and subsequent utilization through transamination may represent a shunt around the oxidation of α-ketoglutarate to succinate in the normal TCA cycle (Fig. 5), since all the enzymes for this shunt pathway are present in the bacteroids. The Gaba aminotransferase-specific activities measured in bacteroid extracts were not adequate, however, to account fully for the rapid initial changes observed in intact bacteroids. This suggests that either other pathways of Gaba utilization are operating or the enzyme activities declined during the extraction process. Gaba appeared to be utilized exclusively through a transamination pathway only when succinate was absent, when it was observed that the Gaba decline was sensitive to the transaminase inhibitor. In the presence of succinate, the decline in Gaba is actually accelerated (Fig. 1), yet this decline is not sensitive to transaminase inhibition. This suggests the operation of a second pathway for metabolism of Gaba that is not catalyzed by pyridoxal phosphate-dependent enzymes, and is stimulated by succinate. The nature of this pathway is currently unknown. Gaba was not exported from isolated bacteroids under any incubation conditions. The possible involvement of two unknown nitrogen-bearing compounds that appeared exclusively in bacteroid extracts is under investigation. Those compounds were observed only in transforming and mature bacteroids (Table 2).

Figure 5 shows the TCA cycle and Gaba shunt pathways that operate in isolated bacteroids. In addition, the malic enzyme pathway produces pyruvate and leads to alanine production. Endogenous pyruvate cannot support nitrogen fixation in isolated bacteroids. Increases in alanine and increased levels of alanine dehydrogenase present in the bacteroids, as compared to free-living cells (McRae et al. 1989c), suggest that this branch pathway is responsible for some intrabacteroidal ammonia assimilation. Figure 6 illustrates the disposition of C_4-dicarboxylate carbon provided to respiring bacteroids. The fact that approximately

75% of the ^{14}C-labeled succinate remaining inside the bacteroids accumulated in the basic fraction and most of the remainder appeared in the acidic fraction indicates that bacteroids synthesize a major portion of the amino acids required for maintenance functions and heme production, which is required for the synthesis of leghemoglobin in the host cell cytoplasm.

Similar results were observed for malate and 2-^{14}C-pyrvate carbon skeletons that were retained. No export of ^{14}C-labeled ketoacids, amino acids, Gaba, or other compounds was detected during the incubation of isolated bacteroids with radiolabeled succinate under any conditions. This confirms that ammonia is the major nitrogen source exported to the host. This process is clearly very efficient, since no accumulation of ammonia above levels observed in anaerobically isolated cells could be shown in nitrogen-fixing bacteroids.

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