Metabolism of glutamate and aspartate in bacteroids isolated from soybean root nodules

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(Received 2 May 1991; revised 2 September 1991; accepted 10 September 1991)

Bacteroids were isolated anaerobically from root nodules of soybean (*Glycine max* L. Merr.) and supplied with ^{14}C - or ^{15}N -labelled glutamate or aspartate under microaerobic conditions where bacteroids retained a high activity of N_2 fixation. Glutamate and aspartate significantly stimulated N_2 fixation (C_2H_2 reduction) and respiration (CO_2 evolution) of isolated bacteroids. They were both utilized as substrates for bacteroid respiration and their nitrogen was rapidly released from the bacteroids as NH_3 . Amino-oxyacetate (AOA) strongly inhibited glutamate utilization, but aspartate utilization was only slightly affected by AOA. Substantial activity of aspartate ammonia-lyase (aspartase) was detected in the cell-free extract of bacteroids. These results suggest that the major pathway of glutamate utilization in bacteroids is transamination to form aspartate followed by direct deamination of aspartate by aspartase. It is also suggested that the 4-aminobutyrate pathway (GABA-shunt) is partly responsible for the catabolism of glutamate by soybean nodule bacteroids. The results are discussed in terms of the possible relationships between C_4 -dicarboxylates and glutamate in carbon and nitrogen metabolism in soybean nodule bacteroids.

Introduction

Soil micro-organisms of the genera *Rhizobium* and *Bradyrhizobium* fix atmospheric N_2 in a symbiotic association with leguminous plants. The bacteroids in the nodule cells utilize plant photosynthate as the energy source and develop the ability to fix N_2 to supply ammonia to plant cells.

Bacteroids differ greatly from free-living *Rhizobium* or *Bradyrhizobium* bacteria with respect to carbon and nitrogen metabolism. One of the most striking differences is that bacteroids can utilize only a very limited range of carbon sources to support their respiration and N_2 fixation. It is believed that the principle carbon sources utilized by bacteroids in the nodule cells are C_4 -dicarboxylates such as malate and succinate. This was first suggested by studies of the utilization of various carbon compounds by bacteroids isolated from nodules,

which indicated the exclusive effectiveness of C_4 -dicarboxylates for respiration and N_2 fixation (Tuzimura & Meguro, 1960; Bergersen & Turner, 1967). Studies of the dicarboxylate-transport (*dct*) mutants of various species of *Rhizobium* suggested that uptake and catabolism of C_4 -dicarboxylate were essential for N_2 fixation by bacteroids (Ronson *et al.*, 1981; Glenn & Brewin, 1981; Finan *et al.*, 1983). More recent evidence demonstrated that bacteroids isolated from soybean nodules oxidized exogenous succinate via the tricarboxylic acid (TCA) cycle, using malic enzyme to produce acetyl-CoA, even under the low oxygen concentrations required for N_2 fixation (Kimura & Tajima, 1989; Tajima *et al.*, 1986, 1990).

On the other hand, involvement of glutamate as a possible carbon source for bacteroids in nodules has been suggested (Kahn *et al.*, 1985; Bergersen & Turner, 1988). We have also studied the metabolism of glutamate and aspartate by bacteroids of soybean nodules because: (1) bacteroids possess an active uptake system for glutamate (Salminen & Streeter, 1987; Udvardi *et al.*, 1988); (2) exogenous glutamate enhances O_2 consumption and N_2 fixation by isolated bacteroids (Bergersen & Turner, 1988); (3) the site of natural ^{15}N enrichment in soybean nodules is predominantly in the bacteroids (Reinero *et al.*, 1983; Yoneyama *et al.*, 1987), suggesting that

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Abbreviations: AOA, amino-oxyacetic acid; GABA, 4-aminobutyric acid; GABA-AT, 4-aminobutyrate aminotransferase; GDC, glutamate decarboxylase; GDH, glutamate dehydrogenase; PBM, peribacteroid membrane; PBU peribacteroid unit; SSDH, succinic semialdehyde dehydrogenase.

bacteroids are supplied N-containing compound(s) from plant cytosol and release their nitrogen through transamination and/or deamination processes (these processes are suggested to cause $^{15}\text{N}/^{14}\text{N}$ discrimination (Macko *et al.*, 1983; Yoneyama *et al.*, 1987); and (4) mutants of *Rhizobium* defective in glutamate catabolism form *Fix⁻* nodules (Kahn *et al.*, 1985).

These data suggest that some amino compounds, most probably glutamate, are also supplied to bacteroids from plant cytosol, as well as C_4 -dicarboxylates. Here we describe how glutamate and aspartate are metabolized in nitrogen-fixing bacteroids, using evidence from ^{14}C and ^{15}N tracer experiments.

Methods

Isolation of bacteroids. Soybeans (*Glycine max* L. Merr. var. Akisengoku) inoculated with *Bradyrhizobium japonicum* A1017 were grown hydroponically as described previously (Kouchi & Yoneyama, 1986). Bacteroids were isolated anaerobically from the nodules of 40–43-d-old plants by a method modified from that of Emerich *et al.* (1979). All isolation procedures were carried out at 0–4 °C in a plastic cabinet under a continuous argon stream, and centrifuge tubes with air-tight caps were used. The oxygen concentration in the cabinet was maintained below 0.02% (monitored by a Model LH-700H oxygen analyser, Toray). Buffers, harvested nodules and acid-washed Polyclar-AT were de-gassed with a vacuum pump and saturated with argon before use. Nodules (30 g) were macerated with 10 g Polyclar-AT in 90 ml 50 mM-potassium phosphate buffer (pH 6.8) containing 0.2 M-sodium ascorbate, 10 mM-dithiothreitol (DTT) and 0.1% bovine serum albumin (fraction V) using a mortar and pestle. The homogenate was filtered through four layers of gauze followed by centrifugation at 500 g for 5 min at 4 °C. The supernatant was further centrifuged at 9000 g for 10 min. The pellet was washed three times with 90 ml of 50 mM-MOPS-KOH (pH 7.4) containing 2.5 mM-MgCl₂, 1 mM-KH₂PO₄ and 10 mM-DTT. The bacteroid pellets were finally suspended in 30 ml of the same medium (8–12 mg protein ml⁻¹ suspension) and stored in aliquots in liquid nitrogen until use. Bacteroids stored in liquid nitrogen retained more than 75% of their initial C_2H_2 reduction activity at least for a few months.

Assays of acetylene reduction and respiration of bacteroids. One ml assay medium (50 mM-MOPS-KOH, 2.5 mM-MgCl₂ and 1 mM-KH₂PO₄, pH 7.4) with or without exogenous substrates was placed in a serum bottle (22 ml volume) with a double rubber cap. The head space was evacuated and then re-filled with argon. This procedure was repeated four times, after which acetylene was introduced to a final concentration of 10% (v/v). Appropriate volumes of oxygen were injected with a gas tight syringe. Reaction was started with an injection of 0.1 ml bacteroid suspension (about 1 mg bacteroid protein) followed by incubation at 28 °C for 20 min with vigorous shaking (on a reciprocating shaker, 190 r.p.m.). The reaction was stopped by injecting 0.1 ml 60% (w/v) H₂SO₄. After a further 20 min incubation to liberate CO₂ dissolved in the medium, 1 ml of the head space gas was analysed by gas chromatography (model GC-3, Shimadzu) with a 2 m stainless-steel column packed with Porapak R. A short column packed with a nickel catalyst (Shimarite, Shimadzu) was inserted between the Porapak R column and a flame ionization detector (FID) and heated at 700 °C in a reaction furnace. Using hydrogen as a carrier gas, CO₂ was reduced to CH₄ and detected by a FID, as well as C₂H₄. All measurements were made for at least duplicate incubations.

Radiorespirometric experiments. Respiratory evolution of $^{14}\text{CO}_2$ from bacteroids fed ^{14}C -labelled substrates was measured with the same incubation system as for the assay of C_2H_2 reduction and respiration. The head space was filled with N₂ instead of $\text{C}_2\text{H}_2/\text{Ar}$ and oxygen concentrations were adjusted to ensure maximum C_2H_2 reduction activity. After incubation, 1 ml of the head space gas was withdrawn and injected into a scintillation vial containing 0.5 ml monoethanolamine-methylcellosolve (1:2, v/v) through a double rubber cap. After the vials had been agitated for 10 min at room temperature, 10 ml toluene scintillator was added and the radioactivity was measured with a liquid scintillation counter.

^{14}C and ^{15}N labelling experiments and amino acid analysis. One ml (about 10 mg protein) bacteroid suspension was incubated with 5 ml assay medium containing ^{14}C - or ^{15}N -labelled substrates in 100 ml serum bottles. The head space was filled with N₂ containing 1.5% (v/v) O₂. After incubation, bacteroids were pelleted immediately by centrifugation at 10000 g for 5 min at 4 °C. The bacteroid pellets were washed once with 10 mM-sodium phosphate buffer (pH 7.4) followed by extraction with 80% (v/v) ethanol in a boiling-water bath for 20 min. After centrifugation, the ethanol extracts were evaporated *in vacuo* and fractionated into neutral, acidic and basic fractions with ion-exchange resins (Wang, 1960).

In ^{14}C -labelling experiments, amino acids in the basic fraction were separated by two-dimensional TLC (Kano *et al.*, 1975) and detected by spraying with o-phthalaldehyde solution (Mori *et al.*, 1977). Individual amino acid spots were scraped off into toluene scintillator to measure radioactivity. Abundances of ^{15}N in amino acids were determined by gas chromatography-mass spectrometry (Kouchi & Yoneyama, 1984). Absolute amounts of amino acids were determined with an automatic amino acid analyser (model L8500, Hitachi).

The concentration of and abundance of ^{15}N in NH₃ in the incubation medium were determined by the indophenol method (Weatherburn, 1967) and emission spectrometry (Yoneyama *et al.*, 1975), respectively, after trapping NH₃ in 0.2 M-HCl by microdiffusion (Yoneyama *et al.*, 1975).

Isolation of peribacteroid-membrane (PBM)-enclosed bacteroids. The isolation procedure for PBM-enclosed bacteroids (peribacteroid unit, PBU) was based on that of Price *et al.* (1987) with some modifications. All isolation procedures were carried out at 4 °C under aerobic conditions and a refrigerated centrifuge (05PR-22, Hitachi) equipped with a swinging bucket rotor (type 05, Hitachi) was used. Nodules (15–20 g) harvested from 40–43-d-old plants were homogenized very gently in homogenization medium with a porcelain mortar (with rough surface) and a wooden pestle. The homogenization medium comprised 100 mM-MOPS-KOH (pH 7.2) containing 0.375 M-mannitol, 10 mM-MgSO₄, 10 mM-EGTA, 10 mM-DTT, 1% (w/v) polyvinylpyrrolidone (PVP-40, Sigma), 0.5% (w/v) bovine serum albumin and 4% (w/v) dextran T40 (Pharmacia). The homogenate was passed through two layers of Miracloth (Calbiochem) followed by centrifugation at 160 g for 15 min to remove unbroken tissues and cell debris. The supernatant was further centrifuged at 680 g for 10 min. The pellet was gently suspended in 10 ml washing medium consisting of 20 mM-MOPS-Bis-Tris propane (pH 7.2), 0.35 M-mannitol, 2 mM-MgSO₄, and 10 mM-KCl. Five ml of the suspension was layered on a discontinuous gradient consisting of 4 ml 45% (v/v), 5 ml 60% and 1 ml 80% Percoll in the washing medium, and centrifuged at 4200 g for 17 min at 4 °C. The PBUs migrated to the 60/80% interphase and 80% layer, and were collected using a wide-bore pipette and resuspended in 10 vols washing medium. The suspension was layered onto 1 ml 80% Percoll and centrifuged at 680 g for 5 min. The PBU pellets on the Percoll cushion were resuspended in 20–30 ml washing medium and centrifuged at 420 g for 5 min. The final pellet of PBUs was suspended in appropriate volumes of washing medium. The PBUs were kept on ice and used within 1 h.

Assays of utilization of malate and glutamate by intact PBUs. One ml PBU suspension was placed in a serum bottle (12.5 ml volume) with a double rubber cap. The bottles were preincubated for 3 min at 27 °C and the reaction was started by injection of 3 µCi (0.11 MBq) [¹⁴C]malate or [¹⁴C]glutamate (final concentrations of 2 or 0.1 mM). After incubation at 27 °C with gentle shaking (30 r.p.m.), the reaction was stopped by injection of 100 µl 60% (w/v) H₂SO₄ followed by 20 min incubation with vigorous agitation. The radioactivity of the head space gas was determined as described above. The same experiment was conducted in parallel using naked bacteroids prepared from the same PBU preparation by 2 min agitation in washing medium with a vortex mixer (Udvardi *et al.*, 1988).

Enzyme assays. Nodules (40 d-old) were homogenized in 100 mM-MOPS-KOH (pH 7.2) containing 0.3 M-mannitol, 10 mM-MgCl₂ and 10 mM-DTT with a mortar and pestle. The homogenates were passed through four layers of gauze followed by centrifugation at 9000 g for 10 min at 4 °C. Precipitated bacteroids were suspended in the same medium and further purified by centrifugation on a discontinuous Percoll gradient (Kouchi & Fukai, 1989). Purified bacteroids were suspended in 50 mM-MOPS-KOH (pH 7.2) containing 10 mM-MgCl₂, 10 mM-DTT and 1% (v/v) Triton X-100, and disrupted in an ultrasonic homogenizer (a Sonifier Model 250, Branson) for 2 min at 60 W output on ice. After centrifugation at 12000 g for 15 min at 4 °C, the supernatants were used as the bacteroid crude extracts. The supernatants after the first centrifugation of the nodule homogenate were further centrifuged at 21 000 g for 10 min at 4 °C and used as the cytosol fraction. The cytosol and bacteroid extracts were passed through a Sephadex G-25 column prior to enzyme assays.

The methods for enzyme assays were as follows: aspartate-oxoglutarate aminotransferase (EC 2.6.1.1) and alanine-oxoglutarate aminotransferase (EC 2.6.1.2), Holtum & Winter (1982); glutamate

dehydrogenase (GDH, EC 1.4.1.3), Dunn & Klucas (1973); glutamate decarboxylase (GDC, EC 4.1.1.15) and succinic semialdehyde dehydrogenase (SSDH, EC 1.2.1.24), Streeter & Thompson (1972); and 4-aminobutyrate aminotransferase (GABA-AT, EC 2.6.1.19), Freney & Gibson (1975). Aspartate ammonia-lyase (aspartase, EC 4.3.1.1) was assayed as described by Tokushige (1985) by determining released ammonium ions by the Nessler method (Le Page, 1964). The protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as a standard.

Results

Table 1 summarizes the effects of various carbon sources on the bacteroid activities (C₂H₂ reduction and CO₂ evolution) as well as on the O₂ concentrations giving the maximum C₂H₂ reducing activity. Succinate, malate, fumarate and oxaloacetate had the most significant effect on the bacteroid activities. C₂H₂ reduction activity of isolated bacteroids increased three times or more by supplying these carboxylates. Some other carboxylates also stimulated the bacteroid activities but were less effective than these four carboxylates, whereas pyruvate and glyoxylate had no significant effect.

Other than the TCA cycle carboxylates, four amino acids, glutamate, aspartate, glutamine and asparagine significantly increased the bacteroid activities. When bacteroids were supplied glutamine or asparagine, they

Table 1. *Effects of various carbon sources on C₂H₂ reduction activity and respiration (CO₂ evolution) of isolated bacteroids*

Data are expressed as means ± SE of eight (for the control values with no substrate) or duplicate measurements. The assay system is described in Methods.

Substrate	Concn (mM)	O ₂ -optimum (%)	C ₂ H ₄ formation [µmol h ⁻¹ (mg protein) ⁻¹]	CO ₂ evolution [µmol h ⁻¹ (mg protein) ⁻¹]
None		0.42	0.11 ± 0.003	0.97 ± 0.002
Acetate	2.0	0.55	0.14 ± 0.02	1.40 ± 0.15
Pyruvate	2.0	0.43	0.10 ± 0.02	1.20 ± 0.12
Succinate	2.0	1.02	0.35 ± 0.06	2.75 ± 0.23
Malate	2.0	0.94	0.33 ± 0.03	2.74 ± 0.44
Fumarate	2.0	0.84	0.42 ± 0.05	3.31 ± 0.29
Oxaloacetate	2.0	0.66	0.38 ± 0.06	2.67 ± 0.38
2-Oxoglutarate	2.0	0.84	0.11 ± 0.01	1.26 ± 0.22
Citrate	2.0	0.81	0.24 ± 0.03	2.18 ± 0.15
3-Hydroxybutyrate	3.0	0.84	0.17 ± 0.02	1.19 ± 0.08
Glyoxylate	2.0	0.41	0.10 ± 0.01	1.10 ± 0.17
Succinic semialdehyde	2.0	0.92	0.28 ± 0.03	2.51 ± 0.32
Glutamate	0.2	0.48	0.16 ± 0.00	1.14 ± 0.18
	2.0	0.74	0.21 ± 0.03	2.02 ± 0.16
Glutamine	0.2	0.76	0.26 ± 0.01	1.69 ± 0.09
	2.0	0.82	0.21 ± 0.02	1.99 ± 0.18
Aspartate	0.2	0.57	0.25 ± 0.05	1.71 ± 0.20
	2.0	0.70	0.20 ± 0.02	2.18 ± 0.27
Asparagine	0.2	0.57	0.25 ± 0.03	1.71 ± 0.11
	2.0	0.80	0.18 ± 0.01	2.18 ± 0.10
4-Aminobutyrate	2.0	0.39	0.14 ± 0.00	0.98 ± 0.13
Alanine	2.0	0.49	0.13 ± 0.02	1.07 ± 0.09

Fig. 1. Effects of glutamate (a, c) and aspartate (b, d), with or without AOA, on C_2H_2 reduction (a, b) and CO_2 evolution (c, d) by bacteroids isolated from soybean root nodules. ($\square$), Endogenous activities (no added substrate); ($\circ$), 2 mM-glutamate or aspartate; ($\bullet$), 2 mM-glutamate or aspartate with 3 mM-AOA. Data are expressed as means of duplicate incubations. Vertical bars indicate $2 \times \text{SE}$. The assay system is described in Methods.

were converted very rapidly to glutamate or aspartate, respectively in the medium (data not shown), suggesting that these amides were utilized by bacteroids mainly after rapid deamidation in the supernatant of the bacteroid suspension.

The effect of amino-oxycetate (AOA) as a specific inhibitor of transamination on glutamate- or aspartate-dependent C_2H_2 reduction and CO_2 evolution was examined (Fig. 1). The addition of AOA (3 mM) in the incubation medium decreased glutamate-dependent bacteroid activities to the endogenous levels but had no effect on aspartate-dependent ones. Similar effects of AOA on the utilization of glutamate and aspartate as respiratory substrates for bacteroids were observed in radiorespirometric experiments (Fig. 2). The addition of AOA resulted in an approximately 60% decrease in $^{14}CO_2$ evolution from glutamate, whereas utilization of aspartate was only slightly affected by AOA.

Table 2 shows the incorporation of ^{15}N into free amino acids in the bacteroids supplied with [^{15}N]glutamate or [^{15}N]aspartate, as well as into NH_3 in the medium. The largest amount of ^{15}N was found in NH_3 in the medium. The abundances and absolute amounts of ^{15}N in NH_3 in the medium were both much higher in the

incubation with [^{15}N]aspartate than with [^{15}N]glutamate. The distributions of ^{15}N in individual amino acids were very similar in aspartate- and glutamate-fed bacteroids. The highest ^{15}N abundance was found in glutamate and 4-aminobutyrate. Since glutamate is the largest pool among bacteroid amino acids (Kouchi & Yoneyama, 1986; Streeter, 1987), most of the ^{15}N in the bacteroids was in glutamate. The amount of label in aspartate was very low even in the bacteroids fed [^{15}N]aspartate. The same experiments were conducted with or without AOA (Table 3). AOA greatly decreased ^{15}N abundance in amino acids in the bacteroids other than exogenously supplied amino acids, indicating that transamination reactions in bacteroids were effectively inhibited by AOA. In the incubation with [^{15}N]glutamate, AOA greatly decreased labelling of NH_3 in the medium, whereas $^{15}NH_3$ liberation from [^{15}N]aspartate was affected much less.

Since the results of the inhibition experiments using AOA suggested the direct deamination of aspartate, the presence of aspartase in bacteroids was examined (Table 4). Cell-free extract of bacteroids passed through a Sephadex G-25 column showed substantial activity of aspartase and, as expected, this activity was not

Fig. 2. Respiratory utilization of [^{14}C]aspartate (a) and [^{14}C]glutamate (b) by isolated bacteroids with (●) or without (○) 3 mM-AOA. Bacteroids (0.1 ml suspension with 1 ml assay medium) were incubated with 2 mM-[U- ^{14}C]aspartate or [U- ^{14}C]glutamate (0.2 μCi , 7.4 kBq) under 0.8% O_2 in N_2 . Data are expressed as means of duplicate incubations. Vertical bars indicate $2 \times \text{SE}$.

Table 2. Incorporation of ^{15}N into free amino acids in the bacterioids and NH_3 in the incubation medium

The bacterioids (1 ml suspension) were incubated with 5 ml assay medium containing [^{15}N]aspartate or [^{15}N]glutamate (2 mM, 52.7 atom%) under 1.5% O_2 in N_2 . Data are expressed as means $\pm$ SE of duplicate experiments. Absolute amounts of ^{15}N [nmol (mg protein) $^{-1}$] are shown in parentheses except values < 0.1 nmol.

Amino acid	Incorporation of ^{15}N (atom%)			
	Aspartate		Glutamate	
	10 min	20 min	10 min	20 min
Asp	5.9 ± 0.57	10.1 ± 2.03 (0.1)	5.8 ± 0.78	10.1 ± 0.19 (0.1)
Thr	3.5 ± 0.14	4.8 ± 0.13	2.8 ± 0.01	4.1 ± 0.80
Ser	0.9 ± 0.21	2.6 ± 0.15	1.3 ± 0.41	2.2 ± 0.36
Glu	20.3 ± 3.08 (2.9)	26.2 ± 1.26 (4.4)	28.5 ± 0.69 (4.4)	32.0 ± 0.09 (5.9)
Gly	1.1 ± 0.01	1.3 ± 0.01	0.9 ± 0.18	1.3 ± 0.07
Ala	6.7 ± 0.21 (0.5)	9.6 ± 0.92 (0.7)	8.9 ± 0.04 (0.6)	11.6 ± 0.13 (0.8)
Val	9.6 ± 0.25 (0.2)	12.1 ± 0.28 (0.2)	9.3 ± 0.03 (0.2)	11.3 ± 0.06 (0.2)
Ile	10.4 ± 1.29 (0.1)	12.1 ± 2.40 (0.2)	15.0 ± 0.20 (0.2)	18.6 ± 0.77 (0.2)
Leu	8.7 ± 0.28 (0.1)	12.0 ± 1.33 (0.2)	11.3 ± 0.35 (0.1)	14.5 ± 0.04 (0.2)
GABA	22.1 ± 0.35	27.7 ± 0.91	28.5 ± 0.63	33.6 ± 0.22
NH_3 in the medium	30.0 ± 1.00 (16.4)	30.5 ± 0.05 (26.7)	21.8 ± 0.30 (6.7)	24.6 ± 0.60 (13.5)

inhibited by AOA. On the other hand, aspartase activity in plant cell cytosol was very low.

Incorporation of ^{14}C from [^{14}C]aspartate, [^{14}C]glutamate and [^{14}C]malate into soluble fractions of bacteroids is shown in Table 5. The highest radioactivity was found in cationic fractions and the largest amount of ^{14}C was present in glutamate in all experiments.

Table 6 shows amino acid concentrations in bacteroids and in the medium after incubation with malate, glutamate or malate plus glutamate. Glutamate was the most abundant amino acid in the bacteroids in all treatments and increased greatly by incubating with glutamate. Alanine in bacteroids also increased slightly by incubating with malate but the concentrations of

Table 3. Incorporation of ^{15}N into free amino acids in the bacteroids fed ^{15}N aspartate or ^{15}N glutamate with or without AOA, and in NH_3 liberated into the medium

The bacteroids (1 ml suspension) were incubated with 5 ml assay medium containing ^{15}N aspartate or ^{15}N glutamate (3 mM, 52.7 atom%) with or without 5 mM-AOA for 20 min under 1.5% O_2 in N_2 . Data are expressed as means $\pm$ SE of duplicate experiments.

Amino acid	Incorporation of ^{15}N (atom%)			
	Aspartate		Glutamate	
	- AOA	+ AOA	- AOA	+ AOA
Asp	5.2 $\pm$ 0.38	12.5 $\pm$ 0.43	8.3 $\pm$ 1.17	3.7 $\pm$ 0.77
Thr	4.0 $\pm$ 0.91	3.9 $\pm$ 0.51	2.5 $\pm$ 0.24	0.1 $\pm$ 0.05
Ser	0.3 $\pm$ 0.02	0.0	0.7 $\pm$ 0.21	0.2 $\pm$ 0.11
Glu	31.8 $\pm$ 0.42	9.2 $\pm$ 0.45	35.0 $\pm$ 0.15	38.7 $\pm$ 0.68
Gly	1.0 $\pm$ 0.10	0.5 $\pm$ 0.30	1.1 $\pm$ 0.06	0.6 $\pm$ 0.06
Ala	7.2 $\pm$ 0.19	3.1 $\pm$ 0.01	10.1 $\pm$ 0.48	1.6 $\pm$ 0.20
Val	10.4 $\pm$ 0.63	2.2 $\pm$ 0.03	10.3 $\pm$ 0.29	3.3 $\pm$ 0.30
Ile	12.3 $\pm$ 1.47	2.0 $\pm$ 1.01	17.1 $\pm$ 0.87	4.8 $\pm$ 0.72
Leu	6.8 $\pm$ 0.02	1.7 $\pm$ 0.41	11.7 $\pm$ 0.03	4.0 $\pm$ 0.50
GABA	26.5 $\pm$ 0.08	4.2 $\pm$ 0.13	32.8 $\pm$ 0.06	4.7 $\pm$ 0.06
NH_3 in the medium	30.2 $\pm$ 0.45	20.8 $\pm$ 0.35	25.3 $\pm$ 0.20	6.2 $\pm$ 0.12

Table 4. Aspartate ammonia-lyase (aspartase) activities in plant cell cytosol and bacteroids of soybean root nodules

The reaction mixture (1 ml) consisted of 100 mM-Tris/HCl (pH 8.8), 100 mM-sodium aspartate, 2 mM- MgCl_2 , 5 mM-KCl and 50 μl crude extract of cytosol or bacteroids. After the incubation at 30 $^\circ\text{C}$ for 30 min, NH_3 released was determined colorimetrically by the Nessler method. The extracts, after boiling for 5 min, were used as a control. Data for duplicate assays are shown.

	Aspartase activity [nmol NH_3 (mg protein) $^{-1}$ min $^{-1}$]	
	Cytosol	Bacteroid
- AOA	5.1 3.9	54.5 59.1
+ AOA (1 mM)	3.8 6.2	59.1 62.5

other amino acids were not significantly affected by exogenous substrates. When bacteroids were incubated with malate, alanine markedly accumulated in the medium, whereas aspartate accumulated in the medium during the incubation with glutamate. The accumulation of aspartate and alanine in the medium increased greatly when bacteroids were incubated with malate plus glutamate.

Enzyme activities relating to glutamate catabolism in the bacteroids were assayed (Table 7). The activities of GDH and aspartate-oxoglutarate aminotransferase were similar in plant cell cytosol and bacteroids on a total protein basis. Glutamate decarboxylase, the first enzyme of the 4-aminobutyrate pathway (GABA shunt) was at

Table 5. Incorporation of ^{14}C into soluble fractions of bacteroids from ^{14}C aspartate, ^{14}C glutamate and ^{14}C malate

The bacteroids (1 ml suspension) were incubated with 5 ml assay medium containing 2 mM- (5 μCi , 0.185 MBq) ^{14}C aspartate, ^{14}C glutamate or ^{14}C malate under 1.0% O_2 in N_2 for 20 min. Data are expressed as means $\pm$ SE of duplicate experiments.

Substrate	Incorporation of ^{14}C ($10^{-3} \times \text{d.p.m.}$)		
	Aspartate	Glutamate	Malate
Neutral	106 $\pm$ 19.6	70.5 $\pm$ 7.9	414 $\pm$ 7.0
Anionic	64.1 $\pm$ 4.4	74.7 $\pm$ 4.5	146 $\pm$ 16.6
Cationic	381 $\pm$ 0.6	609 $\pm$ 14.8	432 $\pm$ 41.2
Cationic fraction			
Asp	3.88 $\pm$ 0.37	3.89 $\pm$ 1.32	7.77 $\pm$ 1.80
Ser	5.92 $\pm$ 0.45	11.2 $\pm$ 2.58	8.52 $\pm$ 0.05
Asn	0.69 $\pm$ 0.13	0.33 $\pm$ 0.14	1.33 $\pm$ 0.94
Glu	192 $\pm$ 11.4	286 $\pm$ 34.1	97.4 $\pm$ 18.1
Gln	2.37 $\pm$ 0.10	1.58 $\pm$ 1.36	2.87 $\pm$ 1.79
Gly	2.13 $\pm$ 0.02	2.12 $\pm$ 0.47	3.18 $\pm$ 1.62
Ala	3.27 $\pm$ 0.18	2.72 $\pm$ 1.18	3.65 $\pm$ 0.40
Val	1.81 $\pm$ 0.09	2.31 $\pm$ 0.27	2.38 $\pm$ 0.25
Leu	3.56 $\pm$ 0.17	3.14 $\pm$ 0.08	7.16 $\pm$ 0.29
GABA	3.84 $\pm$ 1.05	4.56 $\pm$ 1.56	4.54 $\pm$ 0.46

extremely low activity in bacteroids as compared with plant cell cytosol. However, the activities of two other enzymes involved in the GABA shunt, GABA-AT and SSDH, were tenfold higher in bacteroids than in plant cytosol. GABA-AT in bacteroids was more active with pyruvate and oxaloacetate than with 2-oxoglutarate, unlike the situation in cowpea *Rhizobium* sp. MNF2030 (Jin *et al.*, 1990) and *R. meliloti* bacteroids (Miller *et al.*, 1991).

Table 6. Amino acid contents in bacteroids after incubation with malate, glutamate, or malate + glutamate

One ml bacteroid suspension was incubated with 5 ml assay medium with or without exogenous substrates for 20 min under 1.5% O₂ in N₂ and amino acid content was measured (B). The net increase in amino acids in the medium during the incubation was also determined (M). All data are means of duplicate experiments (LSD, least significant difference).

Amino acid	Amino acid content [nmol (mg protein) ⁻¹] after incubation with:									
	None		Malate (2mM)		Glutamate (2mM)		Malate (2mM) + Glutamate (1mM)		LSD (5%)	
	B	M	B	M	B	M	B	M	B	M
Asp	0.3	0	0.6	0	0.5	4.3	0.5	15.1	0.32	0.01
Thr	1.4	0.3	1.2	0.5	1.2	0.5	0.9	0.7	0.34	0.81
Ser	0.4	0	1.1	0.2	0.3	0.2	0.3	0.6	0.22	0.42
Glu	8.7	0	13.1	0	24.9	—	23.2	—	6.12	—
Gly	0.6	0	1.0	0.2	0.5	0	0.6	0.4	0.55	0.01
Ala	1.1	0	2.0	2.2	1.6	0.3	2.5	9.2	0.54	2.69
Val	2.7	2.1	2.1	2.3	2.0	2.2	1.8	2.6	0.45	1.08
Ile	0.7	1.7	0.5	1.4	0.6	1.8	0.5	1.7	0.13	0.57
Leu	0.7	1.7	0.5	1.6	0.6	1.9	0.5	1.9	0.12	0.91
GABA	0.2	0	0.3	0	0.2	0	0.3	0	0.07	—
Lys	4.5	0.6	3.6	0.4	4.1	0.5	3.2	0.5	1.25	1.38
Arg	2.6	0.7	2.0	0.6	2.5	0.6	1.9	0.7	0.95	0.46
Pro	1.6	0.5	1.8	0.9	1.8	1.0	1.5	1.3	0.34	1.16

Table 7. Enzyme activities related to glutamate catabolism in plant cell cytosol and bacteroids of soybean root nodules

Data are expressed as means $\pm$ SE of duplicate assays. The assay system is described in Methods.

Enzyme	Cofactor/ substrate	Enzyme activity [nmol (mg protein) ⁻¹ min ⁻¹]	
		Cytosol	Bacteroid
Succinic semialdehyde dehydrogenase	NAD	0.73 $\pm$ 0.03	34 $\pm$ 3.3
	NADP	0.97 $\pm$ 0.06	32 $\pm$ 1.9
Glutamate decarboxylase		0.12 $\pm$ 0.14	0.64 $\pm$ 0.01
GABA amino- transferase	Pyr	0.12 $\pm$ 0.12	2.1 $\pm$ 0.44
	OAA	0.14 $\pm$ 0.02	2.4 $\pm$ 0.32
	2-OG	0.04 $\pm$ 0.01	0.11 $\pm$ 0.06
Glutamate dehydrogenase		6.6 $\pm$ 0.36	4.1 $\pm$ 0.04
Aspartate- oxoglutarate aminotransferase		120 $\pm$ 11.2	90 $\pm$ 1.9
Alanine-oxoglutarate aminotransferase		89 $\pm$ 4.0	6.6 $\pm$ 0.56

Pyr, pyruvate; OAA, oxaloacetate; 2-OG, 2-oxoglutarate.

Permeability of the PBM to malate and glutamate was indirectly examined by comparing respiratory evolution of ¹⁴CO₂ from ¹⁴C-labelled substrates by intact PBUs with that by naked bacteroids (Fig. 3). The purity of PBUs isolated in this work was comparable to that shown by Price *et al.* (1987) and the disruption of PBUs after 40 min incubation with gentle rocking was almost

negligible as checked by light and electron microscopy (data not shown). Rates of respiratory utilization of [¹⁴C]malate by intact PBUs were about 60% of those by naked bacteroids at both concentrations of 0.1 and 2 mM. On the other hand, evolution of ¹⁴CO₂ from [¹⁴C]glutamate by the PBUs was less than 15% of that by naked bacteroids at a concentration of 0.1 mM, but about 45% of that at 2 mM.

Discussion

The results of ¹⁵N and ¹⁴C tracer experiments suggest that aspartate and glutamate are both actively oxidized by the TCA cycle after release of amino nitrogen, which is rapidly lost from bacteroids as NH₃. The supply of AOA greatly inhibited amino-transfer reactions in bacteroids as demonstrated by the ¹⁵N labelling experiment (Table 3). In addition, AOA had no inhibitory effect on the transport of both glutamate and aspartate in the preliminary uptake assay of [¹⁴C]glutamate and [¹⁴C]aspartate (data not shown). The strong inhibition of glutamate utilization with AOA, therefore, indicates that glutamate catabolism in bacteroids involves a transamination process(es) as an essential step, and GDH in the bacteroids does not operate in the direction of oxidative deamination. Recently, Salminen & Streeter (1990) also suggested that GDH in soybean nodule bacteroids operates *in vivo* in the direction of glutamate formation in spite of the presence of a large pool of glutamate. When [¹⁴C]malate was supplied to bacter-

Fig. 3. Respiratory utilization of [U- ^{14}C]malate (○) and [U- ^{14}C]glutamate (●) by the intact PBUs isolated from soybean root nodules. The PBUs (solid lines) and naked bacteroids (broken lines) were incubated with ^{14}C -labelled substrates (5 μCi , 0.185 MBq) at concentrations of 2 mM (a) or 0.1 mM (b), and $^{14}\text{CO}_2$ evolved was determined. Data are expressed as means of duplicate incubations. Vertical bars indicate $2 \times \text{SE}$. The assay system is described in Methods.

oids, substantial incorporation of ^{14}C into glutamate was found (Table 5), also suggesting the formation of glutamate from 2-oxoglutarate in the bacteroids.

On the contrary, utilization of exogenous aspartate by bacteroids was not significantly affected by AOA. This indicates that the major pathway of aspartate utilization was via the TCA cycle after direct deamination. The presence of substantial activity of aspartase in bacteroids (Table 4) suggests that this enzyme is responsible for the direct deamination of aspartate. Effects of AOA on ^{15}N abundance in NH_3 released from the bacteroids fed [^{15}N]glutamate or [^{15}N]aspartate (Table 3) also strongly suggest the involvement of transaminations in glutamate catabolism but not in aspartate utilization.

Amino-transfer to oxaloacetate is the most probable first reaction in glutamate catabolism in bacteroids because of the presence of relatively high activity of aspartate-oxoglutarate aminotransferase (Table 7; see also Salminen & Streeter, 1990). Nitrogen derived from glutamate would need to be released from the bacteroids by rapid deamination of aspartate. That ^{15}N abundance in aspartate in the bacteroids fed [^{15}N]glutamate was significantly lower than in released NH_3 seems to conflict with this hypothesis. However, it should be noted that very little incorporation of ^{15}N into aspartate in the bacteroids was found even when they were incubated with [^{15}N]aspartate, despite rapid labelling of glutamate and NH_3 released (Tables 2 and 3). Further, the [^{14}C]aspartate feeding experiment resulted in no significant incorporation of ^{14}C into aspartate in the bacteroids (Table 6), although it was actively respired (Fig. 2). Salminen & Streeter (1987) also reported that ^{14}C from exogenously supplied [^{14}C]aspartate was not

incorporated into the bacteroid aspartate pool, although a substantial amount of ^{14}C appeared in CO_2 respired. These findings indicate that the active pool of aspartate may be extremely small and separated from the large inactive pool in the bacteroids. Exogenously supplied aspartate could be catabolized by bacteroids without being incorporated into their major (inactive) aspartate pool; by contrast glutamate was catabolized after incorporation into the large glutamate pool in the bacteroids.

When bacteroids were incubated with both malate and glutamate, large amounts of aspartate and alanine, particularly aspartate, were released from the bacteroids to the incubation medium (Table 6). Aspartate in the medium was not, or only slightly detectable when the bacteroids were fed malate or glutamate alone, and glutamate was not released from bacteroids incubated with malate. These findings also indicate that in bacteroids aspartate is formed by transamination from glutamate, and furthermore suggest that in intact nodules, where C_4 -dicarboxylates are available for bacteroid respiration as more abundant and effective substrates, aspartate need not be imported into bacteroids from plant cytosol. The physiological significance of liberation of alanine from the bacteroids is unclear, but exogenous alanine did not support the bacteroid respiration (Table 1). One of the possible routes of NH_3 liberation from glutamate is a chain of glutamate-oxaloacetate aminotransferase and aspartate-pyruvate aminotransferase with alanine dehydrogenase as suggested in *Rhizobium* sp. MNF2030 (Jin *et al.*, 1990). Even though such a chain of transaminases with a terminal amino acid dehydrogenase may be also partly

responsible in glutamate catabolism in soybean nodule bacteroids, the data in this study strongly suggest that the major pathway of NH_3 liberation from exogenously supplied glutamate is amino-transfer to form aspartate followed by direct deamination of aspartate by aspartase, as discussed above.

One of the major alternative pathways of glutamate catabolism is the 4-aminobutyrate pathway (GABA-shunt). Operation of the GABA-shunt was recently reported in *R. meliloti* bacteroids (Miller *et al.*, 1991). In *B. japonicum* bacteroids, however, it is suggested that the GABA-shunt does not operate because of the lack of GDC activity (Salminen & Streeter, 1990). In the present work GDC activity in the bacteroids was very low but detectable (net radioactivity in GABA formed in a standard GDC assay was about 2.5-fold of control) and activities of two other GABA-shunt enzymes, GABA-AT and SSDH, were more than tenfold higher in bacteroids than in the plant cytosol (Table 7). Very similar values of ^{15}N abundances in GABA and glutamate throughout the labelling period (Table 2) suggest that GABA is formed directly from glutamate in the bacteroids. The low radioactivity of GABA formed from $[^{14}\text{C}]$ glutamate (Table 5) could be due to the extremely low concentration of GABA in the bacteroids (Table 6) and the high activities of GABA-AT and SSDH in the bacteroids. Furthermore, our previous work using a ^{13}C -labelling technique indicated a relatively high turnover rate of the carbons of GABA in bacteroids (Kouchi & Yoneyama, 1986). Thus, the overall results of tracer experiments and enzyme assays suggest that the GABA-shunt may be responsible for a small fraction of the glutamate catabolism of bacteroids. Since exogenous GABA does not stimulate bacteroid activities (Table 1), transport of GABA to bacteroids from plant cytosol is unlikely even though soybean nodule cytosol contains a considerable amount of GABA (Kouchi & Yoneyama, 1986).

A possibility that glutamate is one of the *in vivo* carbon sources for bacteroids can be criticized because the PBM may be impermeable to glutamate. It was observed that the PBM prevented the supply of glutamate to enclosed bacteroids when intact PBUs were incubated with 0.1 mM-glutamate, though free bacteroids possess a high affinity transport system for glutamate (Udvardi *et al.*, 1988). We also examined the utilization of malate and glutamate by PBUs isolated from soybean nodules (Fig. 4). The respiratory evolution of $^{14}\text{CO}_2$ from 0.1 mM- $[^{14}\text{C}]$ glutamate by bacteroids was severely inhibited by the presence of the PBM enclosing the bacteroids. However, when the PBUs were supplied with $[^{14}\text{C}]$ glutamate at a concentration of 2 mM, the respiratory utilization of glutamate by the enclosed bacteroids was about 45% of that by free bacteroids, whereas malate

utilization was also inhibited to about 60% by the presence of the PBM. In soybean nodules estimates of the *in vivo* concentration of glutamate in the plant cell cytosol range from 1 to 10 mM (Kouchi & Yoneyama, 1986; Streeter, 1987; Bergersen & Turner, 1988). Therefore, the PBM may not work as a potential barrier to the supply of glutamate to the bacteroids from plant cytosol within the range of concentrations expected *in vivo* in the cytosol in nodules.

The authors wish to express their thanks to Dr S. Tajima of Kagawa University, Kagawa, Japan for his helpful discussions throughout this study. They also thank Mrs T. Miura for her technical assistance in plant cultivation and nodule harvesting. This work was supported financially by a fund from The Ministry of Agriculture, Forestry and Fisheries, Japan (BMP 91-V-1-1).

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