Involvement of Glutamate in the Respiratory Metabolism of *Bradyrhizobium japonicum* Bacteroids†

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*Bradyrhizobium japonicum* bacteroids were isolated anaerobically and supplied with 14C-labeled succinate, malate, aspartate, or glutamate for periods of up to 60 min in the presence of myoglobin to control the O2 concentration. Succinate and malate were absorbed about twice as rapidly as glutamate and aspartate. Conversion of substrate to CO2 was most rapid for malate, followed by succinate, glutamate, and aspartate. When CO2 production was expressed as a proportion of total carbon taken up, malate was still the most rapidly respired substrate, with 68% of the label absorbed converted to CO2. The comparable values for succinate, glutamate, and aspartate were 37, 50, and 38%, respectively. Considering the fate of labeled substrate not respired, >95% of absorbed glutamate remained as glutamate in the bacteroids. In contrast, from 39 to 66% of the absorbed succinate, malate, or aspartate was converted to glutamate. An increase in the rate of CO2 formation from labeled substrates after 20 min appeared to coincide with a maximum accumulation of label in glutamate. The results indicate the presence of a substantial glutamate pool in bacteroids and the involvement of glutamate in the respiratory metabolism of bacteroids.

A question about symbiotic nitrogen fixation which remains unresolved is what compounds are supplied by the host to the microsymbiont to support the high demand for reducing equivalents in the conversion of N2 to NH4+. Most of the available evidence supports the idea that the tricarboxylic acid cycle acids succinate and malate are the compounds supplied to N2-fixing bacteroids in legume nodules (23). Much of the recent support for this proposition comes from studies showing that bacterial mutants lacking the ability to absorb dicarboxylic acids form nodules that are incapable of N2 fixation (2, 7, 18).

Other groups have studied the kinetics of uptake of various compounds by bacteroids and have identified organic acids as compounds absorbed by active mechanisms (16, 19). Still other groups have studied the ability of various compounds to support respiration or N2 fixation (acetylene reduction) by isolated bacteroids (15, 20, 28). The results of all of these studies support an important role for dicarboxylic acids in bacteroid metabolism and legume nodule function. However, neither of these approaches has involved a complete analysis of the metabolic fate of compounds supplied to bacteroids. We report here experiments in which the goal was to inventory metabolic products. In an early experiment we discovered that [2,3-14C]succinate is very rapidly converted to glutamate in bacteroids, and this led us to study the metabolic fate of malate, glutamate, and aspartate as well.

**Materials and Methods**

**Isolation of bacteroids.** Soybean plants [*Glycine max* (L.) Merr cv. Beeson 80] were inoculated with *Bradyrhizobium japonicum* 61A76 (Nitratin Co., Milwaukee, Wis.) or USDA 110 (Nitrogen Fixation and Soybean Genetics Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Md.) and grown in a greenhouse. Nodules from 37- to 47-day-old plants were chilled (2°C), placed in a glove box under N2 flow, and ground in a mortar with a pestle, with 0.15 M sodium phosphate buffer, pH 7.5, added in 2-ml portions to a final volume of 2 ml of buffer per g of nodules. The homogenate was filtered through two Miracloth disks in a 50-ml syringe into a 30-ml centrifuge tube. The tube was capped tightly and centrifuged at 6,000 × g for 10 min. The tube was opened in the glove box, the supernatant was discarded, and the pellet was washed twice by pipetting 1 ml of the buffer over it. The pellet was resuspended, and the mixture was again filtered through Miracloth disks to remove any unsuspended bacteroids and adjusted to a final concentration of 2 ml of buffer per g of nodules. This suspension was used in the studies on uptake and metabolism. The protein concentration of the bacteroid suspension was determined by the method of Lowry et al. (10).

**Uptake and metabolism.** All substrates were purchased from Amersham Corp. and were uniformly labeled except succinate, which was labeled in the 2 and 3 or the 1 position. [2,3-14C]- and [1-14C]succinate were mixed to give equal radioactivity in each carbon, providing a substrate which was, statistically equivalent to [U-14C]succinate.

Reaction mixtures (total volume, 2.0 ml) were made up in 15-ml Corex centrifuge tubes (15 [inner diameter] by 100 mm) and contained 1 ml of bacteroids, 0.2 ml of 0.4 mM myoglobin (Sigma Chemical Co.) oxygenated as described previously (16), and 0.3 ml of 0.15 M sodium phosphate buffer, pH 7.5. The tubes were flushed with N2 and sealed with a serum stopper with an attached cup containing a filter paper wick impregnated with 70 μl of 10% KOH. A sample (1.4 ml) was removed from the gas phase and replaced with 1.4 ml of Ar-O2 (80:20), giving a final concentration of 2% O2 in the gas phase. Buffered substrate (0.5 ml; 1 μmol at 1 μCi/μmol) was then injected into the reaction mixture, and the tubes were shaken in a rotary shaker at 250 rpm. Incubation was terminated by placing the tubes in an ice bath. The stoppers were quickly removed, and the tubes were centrifuged at 25,000 × g for 10 min. The supernatant was discarded, and the pellet was washed twice by pipetting 1 ml of the cold buffer over it and discarding the washes. The

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pellets were then extracted two times with 80% ethanol, and the ethanolic extracts were combined and dried in a flash evaporator. The residues were then evaporated in 2.0 ml of H2O. The aqueous samples were applied to Dowex 50 H+ and Dowex 1-formate columns (0.7 cm diameter by 2 cm bed height) in tandem. The neutral fraction was eluted with H2O. The columns were then separated, and amino acids were eluted from the Dowex 50 H+ column with 2 N HCl and the organic acids from the Dowex 1-formate column with 4 M HCOOH. The eluates were evaporated to dryness and taken up in 1 ml of 80% ethanol. Portions of the samples were counted in a cocktail containing 24 g of 2,5-diphenyloxazole (PPO) and 1 g of 1,4-bis(5-phenyloxazolyl)benzene in 2 liters of toluene plus 2 liters of Triton X-100. The KOH-impregnated filter paper was assayed for radioactivity with a PPO-toluene scintillation cocktail consisting of 6 g of PPO per liter of a mixture of toluene and methoxyethanol (2:1).

The amino acids were separated by thin-layer chromatography. Each plate was spotted with 5 μl of amino acid standard containing 9.1 μmol of each of 15 amino acids per ml and 20 μl of concentrated sample from the amino acid fraction. Chromatography and analysis of the spots were carried out as described previously (22).

Zero-time values were obtained and subtracted from all values to give the results reported in figures and tables. In calculations, we assumed that 1 mol of succinate yielded 4 mol of CO2. Nanomoles of substrate in bacteroids and in respired CO2 were calculated by assuming a constant specific radioactivity of 1 μCi/μmol in endogenous substrate. Although the endogenous substrate levels were probably quite small, any endogenous substrate, e.g., succinate, would have lowered the specific activity of [14C]succinate and led to underestimation of the actual rates. Thus, the reported rates represent minimum values.

Incorporation of radioactivity into the ethanol-insoluble fraction of bacteroids was evaluated by incubating insoluble residue with 0.3 N KOH at 37°C for 24 h. Radioactivity solubilized by KOH after bacteroids were fed with labeled malate or glutamate for 60 min amounted to 20% of the total radioactivity taken up by the bacteroids. Characterization of the ethanol-insoluble radioactivity was not pursued in these studies, and this fraction is not considered in the results presented here. Thus, total uptake is defined as the sum of the amount of substrate converted to CO2 and 80% ethanol-soluble compounds.

RESULTS

Freedom of the bacteroid preparations from contaminating mitochondria was established in two ways. First, malate dehydrogenase activity of sonicated bacteroids was not inhibited by inhibitors of mitochondrial malate dehydrogenase activity (29), indicating that the preparation was virtually free of mitochondria (S. O. Salminen and J. G. Streeter, Plant Physiol., in press). Second, bacteroid cytochrome oxidase is insensitive to CO2, whereas mitochondrial cytochrome oxidase is highly sensitive (1). A cytochrome oxidase assay (5) of the same sonicated bacteroid preparation gave no evidence of CO2 sensitivity.

In a preliminary experiment we also compared the rate of CO2 formation from succinate with 0.04 mM myoglobin (Sigma Chemical Co.), 0.05 mM soybean leghemoglobin, or no O2 carrier (4). Both myoglobin and leghemoglobin treatment resulted in significantly greater CO2 evolution than the control, and there was essentially no difference between the hemoproteins; myoglobin (0.04 mM) was used in all subsequent experiments.

FIG. 1. Uptake of [14C]-labeled succinate (○), malate (△), glutamate (□), and aspartate (●) by B. japonicum bacteroids. All substrates were uniformly labeled except succinate, which was labeled in the 2 and 3 carbons. Results for a 60-min incubation with [U-14C]succinate are indicated (○). Nanomoles of substrate in ethanol extracts of bacteroids and in respired CO2 were calculated by assuming a constant specific radioactivity of 1 μCi/μmol in endogenous substrate. In the figure, total uptake represents the sum of these two components; the vertical bars represent two standard errors, and each curve represents a different bacteroid preparation. The total bacteroid protein in each reaction mixture was 7.6 mg for succinate, 7.1 mg for glutamate, 9.3 mg for malate, and 12.4 mg for aspartate feeding.

Bacteroids prepared and incubated as described above were checked for nitrogenase activity by an acetylene reduction assay (11) in a reaction mixture that was the same as that used with radioactive substrates. The 2% O2 concentration was not optimal (11), but the rates, after an initial lag period, were linear for at least 90 min. The rate in the absence of exogenous substrates was 74 ± 1 mol/g of nodule (fresh weight) per h, comparable to the rates reported elsewhere for this O2 concentration (11). The addition of 1 μmol of malate or glutamate resulted in a 25% increase in the rate of C2H2 reduction. This level of stimulation of nitrogenase is low, but the substrate concentration used here (0.5 mM) is less than that generally used (5 mM). No effort was made in the present study to establish the optimal conditions for nitrogenase activity.

The uptake of aspartate was linear for 1 h, whereas the rate of succinate, glutamate, and malate uptake appeared to decline after 20 min (Fig. 1). The dicarboxylic acids were taken up three to five times faster than the amino acids. The total uptake in 60 min represented 23% of the succinate in the reaction mixture. The corresponding percentages for malate, glutamate, and aspartate were 20, 11, and 8%, respectively. Carbon dioxide was evolved from malate much faster than from any other substrate (Fig. 2). The rate of CO2 evolution increased with longer incubation times, the rate after 20 min being clearly greater than the rate before 20 min...
(Fig. 2). When the radioactivity in bacteroids incubated with [14C]succinate or [14C]malate was analyzed, very little radioactivity was found in the organic acid fraction at any incubation time. Virtually all of the recovered radioactivity was in the amino acid fraction, with about 72% of the label in glutamate (data not shown). The fate of aspartate and glutamate label in bacteroids was similar to that of malate and succinate.

A minor concern with these initial experiments was that different bacteroid preparations were used for each substrate. Also, [2,3-14C]succinate had been compared with other substrates which were uniformly labeled. Therefore, all four substrates were compared with the same bacteroid preparation, with a single 60-min incubation time and [U-14C]succinate (Table 1). The results agreed with the data in Fig. 1 and 2. Succinate was taken up most rapidly, whereas malate was the most rapidly respired substrate. Relative to total uptake, 68% of the malate was converted to CO2, whereas the corresponding value for succinate was 37%. Most of the difference was accounted for by the amino acid fraction; 48% (12.8 nmol/mg of protein per h) of the succinate taken up was in this fraction, whereas only 26% of the malate was converted into amino acids. Glutamate and aspartate were taken up more slowly than succinate, but glutamate had a greater relative rate of conversion to CO2 than succinate. In fact, the major fate of glutamate appeared to be conversion to CO2.

Thin-layer chromatography of the amino acid fraction indicated that glutamate was consistently the most highly labeled amino acid (Table 2). With succinate feeding, the radioactivity in the glutamate spot (6.45 nmol/mg of protein per h) represented 67% of the radioactivity recovered from the plate (total recoveries were ca. 80 to 90% of the radioactivity spotted); for malate, glutamate represented 80% of radioactivity, for glutamate 97%, and for aspartate 88%. The labeling of glutamate was quite rapid; there was little further increase in glutamate radioactivity after the first 20 min (Fig. 3). Only when aspartate was fed was there a continued increase in 14C-labeled glutamate. The marked differences among substrates in the labeling of glutamate (Fig. 3) seem to indicate that the bacteroids were depleted of glutamate when feedings were initiated and that the capacity for accumulation of glutamate is substantial. Alanine and, to a lesser degree, aspartate were the only other amino acids showing significant labeling (Table 2). In time course studies, supply-

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**TABLE 1. Conversion of 14C-labeled substrates to CO2 and other metabolites by B. japonicum bacteroids**

<table>
<thead>
<tr>
<th>Radioactive substrate supplied</th>
<th>Distribution of label (nmol/mg of protein per h, mean ± SE)</th>
<th>Converted to CO2</th>
<th>Ratio, converted to CO2/total uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid fraction</td>
<td>Organic acid fraction</td>
<td>Total uptake&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>12.8 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>26.5 ± 0.4</td>
</tr>
<tr>
<td>Malate</td>
<td>5.7 ± 0.7</td>
<td>0.4 ± 0.1</td>
<td>21.8 ± 0.7</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5.8 ± 0.3</td>
<td>0</td>
<td>12.2 ± 0.6</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3.8 ± 0.2</td>
<td>0.1 ± 0</td>
<td>7.2 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Subsamples of the same bacteroid preparation were used for the comparisons of all four substrates in a 1-h incubation. The total protein per reaction mixture was 8.6 mg.

<sup>b</sup> All four substrates were uniformly labeled. [U-14C]succinate consisting of a mixture of [2,3-14C]- and [1-14C]succinate, and all substrates were adjusted to a specific radioactivity of 1 μCi/μmol with unlabeled substrate.

<sup>c</sup> The nanomoles of substrate converted into each fraction were calculated by using the ratio of 1 μCi/μmol in the applied substrate. Means and standard errors for duplicate samples are given.

<sup>d</sup> For all substrates, labeling of the neutral fraction was essentially nil and is not shown. Total uptake does not include 14C incorporated into ethanol-insoluble compounds.
FIG. 3. Accumulation of label in glutamate from the substrates supplied to B. japonicum bacteroids. Nanomoles of glutamate were calculated by assuming a specific radioactivity the same as the substrate supplied. See the legend to Fig. 1 for symbols and experimental conditions.

ing bacteroids with malate, aspartate, or glutamate resulted in a transient peak in the labeling of alanine, giving maximum levels in the 20-min samples of 1.33 ± 0.13 nmol/mg of protein (mean ± standard error) for malate feeding, and values of 0.84 ± 0.01 nmol for aspartate feeding and a trace peak for glutamate feeding. [2,3-14C]Sucinate in contrast has 1.91 ± 0.49 nmol of substrate converted to alanine after 20 min. After 60 min the value was 2.80 ± 0.16 nmol.

DISCUSSION

Very few previous studies are comparable to those reported here. The metabolism of 14C-labeled pyruvate, succinate, and acetate by B. japonicum bacteroids has been reported for periods of 1 to 24 h (21). However, labeling of individual compounds in bacteroids was not reported. Glutamate was included as a substrate in one experiment but was only poorly absorbed by bacteroids (21). A problem in comparing our work with the older literature is that the O2 concentration was not controlled in some previous studies (21, 28). It is now clear that bacteroids, which are conditioned to a low-O2 environment, are markedly influenced by O2 concentration in their metabolism of carbon compounds (4, 15, 25, 26). In a recent study with low O2 concentrations, [2,3-14C]Sucinate was incorporated mainly into tricarboxylic acid cycle acids (24), with little or no 14C in amino acids. We suspect that the discrepancy between their results and ours may be explainable on the basis that they used an Ar-O2 mixture, whereas we used N2-O2. Provided with N2-O2, bacteroids should be actively fixing N2 (4), which would not be the case with Ar-O2. The C2H2 reduction in our samples continued for the duration of the experiments (60 min), and the rates were in agreement with previous data (11).

The comparative experiment with the same bacteroid preparation (Tables 1 and 2) confirmed the features of the time course experiments (Fig. 1, 2, and 3). (i) Succinate was more readily converted to amino acids than was malate. In fact, about half of the succinate metabolized was converted to amino acids. (ii) Malate was more readily converted to CO2 than was succinate. This result suggests that malate entering the bacteroid is metabolized via a pathway different from that involved in the metabolism of malate formed in the cell. There is increasing evidence for the aggregation of enzymes in mitochondria (6, 17), as well as in bacteria (3). The resulting “channeling” of metabolites can lead to specific pathways being favored; perhaps such an arrangement exists in Rhizobium bacteroids. (iii) There was essentially no radioactivity in the organic acid fraction. This result indicates that organic acid pools in bacteroids are very small. Organic acids taken up are quickly converted either to CO2 or to amino acids. (iv) Glutamate and aspartate were taken up slowly relative to organic acids, but both were readily converted to CO2. In fact, essentially the only fate of glutamate was conversion to CO2, and glutamate was more readily converted to CO2 than was succinate relative to the total uptake (Table 1).

Our suggestion of the importance of glutamate in bacteroid metabolism is supported by two other lines of evidence. When 15N2 was supplied to intact soybean nodules and the distribution of 15N in bacteroids was examined after 5 or 10 min, most of the label was in glutamate (14). Although the total quantity of 15N in bacteroids was small, the level of enrichment of glutamate in bacteroids was as great as that in the cytosol (14). Secondly, a mutant of B. japonicum which lacks glutamate synthase activity could be induced to fix N2 in vitro but had a Fix− phenotype in nodules (12). It has recently been suggested that this result may indicate a requirement for glutamate synthesis in bacteroids (9), but it is not clear why transamination could not supply glutamate in these bacteroids.

Several attractive arguments suggesting that glutamate may serve as an important source of carbon for bacteroids have recently been put forward (8). The hypothesis is supported by the observation that when glutamate is supplied to rhizobia induced to fix N2 in vitro, NH4+ assimilation is inhibited and the excretion of NH4+ is promoted (13, 27). However, this hypothesis is not supported by studies showing that Rhizobium mutants unable to absorb dicarboxylic acids form Fix− nodules (2, 7, 18), and this is especially true when the mutant is able to grow in culture with glutamate as the source of carbon (2).

We suggest that both dicarboxylic acids and glutamate are respiratory substrates of Rhizobium bacteroids. Several of our observations are consistent with some involvement of glutamate in bacteroid respiration. For example, the increased rate of 14CO2 production (Fig. 2) appeared to coincide with the leveling off of the radioactivity in the glutamate pool (Fig. 3) when succinate, malate, and glutamate were supplied. The changes occurred in the absence of any marked change in uptake rate, at least for malate and glutamate (Fig. 1). Thus, the overall time course results indicate a rapid funneling of carbon into the glutamate pool and concomitant conversion of glutamate to CO2, the latter process reaching equilibrium with the former after the labeling of the glutamate pool has reached a certain level. The
fact that another amino acid, aspartate, is rapidly converted to glutamate emphasizes the importance of glutamate in bacteroid metabolism.

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LITERATURE CITED


