Succinate Degradation through the Citric Acid Cycle in *Bradyrhizobium japonicum* J501 Bacteroids under Low Oxygen Concentrations

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The succinate metabolizing pathway under low oxygen concentrations in soybean nodule bacteroids was surveyed by pulse-chase experiments using [2,3- ^{14}C]succinate and by radiorespirometry. Oxidation of exogenous succinate through the complete citric acid cycle was strongly suggested. Amino acid and sugar formations from exogenous succinate were also observed in the bacteroids. Addition of succinate to the bacteroids increased both the NAD^+/NADH ratio from 1.8 to 3.0 and the adenylate energy value from 0.25 to 0.53.

C_4 -dicarboxylic acids, like malic acid or succinic acid, are the most probable *in vivo* substrates for nodule bacteroids.^{1,6,12)} While it has been shown that cultured *Rhizobium* has a functional citric acid cycle for the oxidation of C_4 -dicarboxylic acids,^{4,7)} the nature and control of the cycle in the bacteroids has not been studied well.

When the profiles of the dicarboxylic acid degradation in aerobically isolated nodule bacteroids were analyzed by a radiorespirometric technique under 20% O_2 , both the citric acid cycle and glyoxylic acid cycle were suggested to be operative.¹⁴⁾ In their experiment, however, the nitrogenase as a major consumer of ATP and the reductant in the bacteroids was inactive because the enzyme system is extremely labile to O_2 .

We already reported that organic acid degradation in the bacteroids was negligible in 2% O_2 , and the incubation system did not accumulate anaerobic metabolites like ethanol

or lactic acid under such low oxygen concentrations,^{16,17)} indicating that the bacteroids can metabolize succinate only under oxidative conditions. Addition of Lb, a hemoprotein supporting oxygen transfer in the nodules, to the incubation system restored the organic acid degrading activity even with such low O_2 , and in this condition the amount of reduced acetylene (nitrogenase activity) depended on that of added succinate.¹⁶⁾

In this experiment the anaerobically isolated bacteroids were pulse-labeled with ^{14}C -succinate under the conditions that the nitrogenase system was operating (100 μM Lb O_2 and 2% O_2/Ar), and the fate of ^{14}C was monitored. In addition, radiorespirometry was used to elucidate the pathway for succinate oxidation. Changes in the redox state of NAD^+ and adenylate energy charge value were also measured to analyze the relationship between carbon metabolism and energy metabolism.

Materials and Methods

Biological materials. Soybean seeds were infected with *Bradyrhizobium japonicum* NIAS J501 and grown in water culture as already described,¹⁵⁾ and nodules from 6- to 7-week-old plants were used for bacteroid and Lb preparations. Both bacteroids and Lb preparations obtained by a series of chromatographies and differential centrifugations,²⁾ respectively, were stored in liquid nitrogen in a Dewar container.

For preparing sample extracts for nucleotide measurement, nodules or roots were crushed in liquid nitrogen immediately after detaching and were ground in a pre-chilled mortar with a pestle. The powder (0.5 g fresh weight) of frozen tissues was used for biochemical assays.

For organic acid measurement the isolated bacteroids were homogenized quickly at 0°C with 5 ml of 1 M perchloric acid. The homogenate was mixed with one ml of 0.1 M Hepes buffer, pH 7.5, and was neutralized carefully with 3 N KOH following a centrifugation at $3,000 \times g$ for 10 min. The supernatant was used for organic acid measurement.

Biochemical assays. Citrate,⁹⁾ 2-oxoglutarate,⁸⁾ succinate,²⁰⁾ fumarate,³⁾ and malate²¹⁾ in bacteroids were measured enzymatically. Elimination of fluorescent compounds in the nodules by a small active charcoal column (0.7 × 2 cm) was necessary for the fluorometric assay. Recovery ratios of organic acids from the column were measured by passing through a known amount of organic acids.

NAD⁺ and NADH in bacteroids were measured by enzymatic cycling methods as Passonneau and Lowry reported.¹⁰⁾

AMP, ADP and ATP in bacteroids were measured by HPLC.¹⁸⁾

Pulse-chase experiment. The incubation system contained 25 μ Ci of [2,3-¹⁴C]succinate, 640 nmol of succinate, and 2 μ mol of Lb in 2.5 ml of 10 mM hepes buffer, pH 7.0. The system was bubbled with 2% O₂/Ar for 15 min at 28°C, and the gas was introduced into two traps containing monoethanolamine-methanol (1:1, v/v) solution. To eliminate the effects of NH₃ that might be accumulated during the incubation and inhibit ATP formation, O₂/Ar was used instead of O₂/N₂. The reaction was started by injecting the bacteroid suspension (100 mg bacteroids in 1.5 ml hepes buffer pH 7.0) through a serum rubber septum. After 5 min of incubation with ¹⁴C-succinate, the incubation system was changed to that of cold succinate by centrifugation (10,000 × *g*, 5 min). Air-tight centrifuge tubes were used to keep low oxygen tension in the buffer system, and it took 15 min at 4°C to change the incubation system. Then the chase treatment was started by incubating the suspension again in the same conditions as described above except the substrate was cold succinate. Every 10 min, one ml of the suspension and 0.1 ml of the

ethanolamine solution in the two traps were collected and were used for biochemical and radioactive assays.

For fractionations of bacteroid extract, the method of Redgwell¹¹⁾ was basically used, except that keto acids in the organic acid fraction were measured after converting those to corresponding amino acids.¹⁹⁾

For separating organic acids by HPLC an anion exchange resin column (0.46 × 25 cm) packed with MCI gel CDR-10 (Cl-form, Mitsubishi Kasei Co.) was used. The column was eluted with 0.15 M NaCl (adjusted the pH to 3.1 with phosphoric acid) at 55°C, and major organic acids of citric acid cycle were separated in 30 min.¹⁷⁾

Sugars were separated by HPLC with a CK08S column (H⁺-form, 0.8 × 25 cm) and with CDR-10 column (borate-form, 0.46 × 25 cm). Sample fractions were put onto the CK08S column, and the column was eluted with water at room temperature resulting in separation by molecular weight. Further separations of the disaccharide fractions that were separated by CK08S column were done with a CDR-10 (borate form) column by eluting with 0.2 M borate buffer, pH 9.0, at 55°C.

Radiorespirometric experiment The apparatus for the reaction system was basically that of the *Pulse-chase experiment*, and 5 ml of 20 mM Hepes buffer pH 6.5 containing 600 nmol of Lb, 2.4 μ mol of succinate, and 0.5 μ Ci of various ¹⁴C-organic acids was bubbled continuously with 2% O₂/Ar at 30°C for 15 min. The bacteroid suspension (50 mg dry weight/ml 20 mM Hepes buffer, pH 6.8) were then injected into the incubation system, and the reaction was started to trap the released ¹⁴CO₂. Every 10 min 100 μ l of the solution in the traps was collected and the radioactivity was measured.

Results

During the pulse-labeling period, approximately 0.25 μ Ci of ¹⁴C (about 1% of total ¹⁴C-succinate in the incubation system) was incorporated into the bacteroids, and after centrifugation to change the incubation system to that for chase treatment, the radioactivity in the supernatant decreased to 2.4% of the initial value (Fig. 1). No ¹⁴CO₂ was released during the pulse (5 min) and the following centrifugation (15 min) periods, and this stalling of respiration was suggested to be due to the low temperature (4°C) during the centrifugation. ¹⁴CO₂ release started at the beginning of the chase treatment, and 40% of the ¹⁴C in the incubation system was recovered as ¹⁴CO₂ in 30 min. Though the ¹⁴C content in the bacteroids decreased very slowly, the decrease of

Fig. 1. Distribution of ^{14}C among Supernatant (●), Bacteroids (○), and $^{14}\text{CO}_2$ (■) Fractions during the Chase Treatment.

The incubation was started by addition of the bacteroids to the incubation system with cold succinate.

Fig. 2. Distribution of ^{14}C among Amino Acid (□), Organic Acid (●), Keto Acid (■), and Neutral (○) Fractions during the Chase Treatment.

The incubation was started by addition of the bacteroids to the incubation system with cold succinate.

^{14}C in the supernatant was mainly recovered in the CO_2 fraction.

When the bacteroids were fractionated to amino acids, organic acids, keto acids, and neutral fractions, only the organic acid fraction decreased its radioactivity rapidly during the chase (Fig. 2). Other amino acid and neutral fractions kept their radioactivities after spontaneous incorporation of ^{14}C into those fractions at the beginning of the incubation (Fig. 2). The keto acid fraction contained negligible radioactivity.

When the organic acid fraction was analyzed by HPLC, ^{14}C was mainly localized in

Fig. 3. Separation of ^{14}C in the Organic Acid Fraction of the Bacteroids at Various Incubation Times by HPLC. Succinate, malate, citrate, fumarate, and 2-oxoglutarate were eluted at fraction numbers 13, 15, 28, 39, and 43, respectively.

two peaks (P-1 and P-2) at all the incubation times (Fig. 3). The retention times of the peaks corresponded to those of sugar acids like glucuronic acid and of succinate+malate.

When the first peak of ^{14}C (Fig. 3, P-1) was passed through a Sephadex G-25 column (1.2×20 cm) the radioactivity eluted at the void volume. The radioactive fractions were pooled, passed through a SP-Sephadex column, and hydrolyzed with conc H_2SO_4 (90°C , 30 min) after condensing *in vacuo*. When the hydrolysate was put on a Sephadex G-25 column again, the elution pattern of radioactive fractions was of low molecular weight compounds. HPLC analysis with a cation exchange column (MCI gel CK08S, Mitsubishi Kasei Co.) of the hydrolysate showed the elution position of ^{14}C was not of crotonic acid (monomer of poly- β -hydroxybutyrate), but was of monosaccharides like glucuronic acid

(data not shown). This detail is under investigation in our laboratory.

Rechromatography of the second ^{14}C peak (P-2) at 20 min showed that 90% of the ^{14}C was of succinate and another 10% was of malate. Similar data were obtained at 30, 40, and 50 min. Figure 3 also showed that negligible radioactivities were accumulated in other organic acids of the citric acid cycle. No ^{14}C -ethanol or ^{14}C -lactic acid was detected.

The neutral fractions of each incubation time were pooled, condensed, and analyzed by HPLC with a CK08S column (H-form). In all incubation times ^{14}C in the neutral fraction was eluted as one peak, and the elution position was of neutral disaccharides like maltose or sucrose. When the radioactive eluates from the CK08S column were pooled, condensed, and analysed with a CDR-10 column (borate form) as described in Materials and Methods, ^{14}C was eluted as one peak and the elution position corresponded to that of maltose (data not shown).

When the changes in pool sizes of citrate, 2-oxoglutarate, succinate, fumarate, and malate in the bacteroids incubated under the same conditions as in the chase experiment were measured, those major organic acid contents did not change extensively except for succinate during the incubation (Table I). After 10 min of incubation the succinate, fumarate, and citrate pools showed almost constant values.

Table I. CHANGES IN VARIOUS ORGANIC ACID CONTENTS DURING THE INCUBATION WITH EXOGENOUS SUCCINATE

Organic acid assayed	Chase incubation time (min)			
	Control	0.5	10	20
Citrate	110	56	66	77
2-Oxoglutarate	87	56	68	22
Succinate	170	490	170	160
Fumarate	38	70	130	110
Malate	350	520	660	590

The values (nmol/dry weight gram) were expressed as means of duplicate assays. As the control the bacteroids were injected into the incubation system containing 0.5 M PCA.

These data show the bacteroids can synthesize amino acids and sugars from succinate. The presence of almost all the ^{14}C in succinate (P-2), though the flow of ^{14}C from ^{14}C -succinate to $^{14}\text{CO}_2$ was vigorous, suggested that the metabolically active organic acid pool was small and separated from the large inactive one in the bacteroids.

Since the ^{14}C content in major organic acids of citric acid cycle was too small to trace the metabolic pathway (Fig. 3), radiorespirometric technique was used. Figure 4a compares the $^{14}\text{CO}_2$ formation rate from [1,4- ^{14}C]succinate and that from [2,3- ^{14}C]succinate. Figure 4b also compares the values from [1- ^{14}C]acetate and from [2- ^{14}C]acetate. Both clearly showed ^{14}C in carboxyl groups was incorporated into CO_2 much faster than ^{14}C in methylene groups, supporting the citric acid cycle operation in bacteroids. In addition, fluoroacetate (the precursor of fluorocitrate as a inhibitor of isocitrate dehydrogenase, 0.5 mM) and malonate (an inhibitor of succinate dehydrogenase, 5 mM) in the incubation system decreased $^{14}\text{CO}_2$ formation as shown in Fig. 5a

Fig. 4. Evolution of $^{14}\text{CO}_2$ from Various ^{14}C -Organic Acids.

The incubation was started in Fig. 4a by the injection of 0.5 μCi of [1,4- ^{14}C]succinate (○) and [2,3- ^{14}C]succinate (●), and in Fig. 4b by the injection of 0.5 μCi of [1- ^{14}C]acetate (○) and [2- ^{14}C]acetate (●).

Fig. 5. Effects of Various Metabolic Inhibitors on Evolution of $^{14}\text{CO}_2$ from ^{14}C -Organic Acids.

The incubation was started in Fig. 5a by the injection of $0.5 \mu\text{Ci}$ of $[1,4\text{-}^{14}\text{C}]$ succinate (○) and $[1,4\text{-}^{14}\text{C}]$ succinate + 0.5 mM fluoroacetate (●), and in Fig. 5b by the injection of $0.5 \mu\text{Ci}$ of $[1,4\text{-}^{14}\text{C}]$ succinate (○) and $[1,4\text{-}^{14}\text{C}]$ succinate + 5 mM malonate (●).

Table II. CHANGES IN THE REDOX STATES OF PYRIDINE AND ADENINE NUCLEOTIDES DURING INCUBATION WITH EXOGENEOUS SUCCINATE

	Chase incubation time (min)				
	Control	0.5	10	20	30
NAD ⁺	201	—	249	159	192
NADH	110	—	82	72	84
NAD ⁺ /NADH	1.8	—	3.0	2.2	2.3
Energy charge	0.25	0.53	0.29	0.30	0.33

The values (nmol/dry weight gram) were expressed as means of duplicate assays. As the control the bacteroids were injected into the incubation system containing 0.5 M PCA.

and 5b. The data supported the idea of the contribution of citric acid cycle enzyme activities for $^{14}\text{CO}_2$ formation in the bacteroids.

Changes in the redox state of NAD⁺ and AEC values were analyzed during the incubation under the same condition to the chase experiment. Table II shows that the NAD⁺/NADH ratios of the stored bacteroids (control) was 1.8. The value increased to 3.0

just after the bacteroids were injected into the incubation system, and then the values stayed around 2.2.

The AEC value of isolated bacteroids was 0.25, at the 0.5 min of incubation the value increased to 0.53, and then went down to 0.29, followed by a gradual increase to 0.33 (Table II).

Discussion

Figure 1 shows that the bacteroids actively metabolized 40% of total $[2,3\text{-}^{14}\text{C}]$ succinate in the incubation system to $^{14}\text{CO}_2$ during the chase treatment (30 min). Though ^{14}C -amino acids and ^{14}C -sugars were spontaneously synthesized from the ^{14}C -succinate added, radioactivities in those fractions kept their initial values during the incubation, suggesting that the metabolism of those compounds in the bacteroids shifted to their synthesis. Only the organic acid fraction decreased its specific radioactivity linearly with time (Fig. 2 and Table I), indicating the exogenous $[2,3\text{-}^{14}\text{C}]$ succinate was converted to $^{14}\text{CO}_2$ mainly through the organic acid fraction in the bacteroids. Recently Kahn *et al.*,⁵⁾ Yoneyama,²²⁾ and Kouchi⁶⁾ reported that exogenous amino acids could support both the bacteroids' respiration and acetylene reduction activities. Figure 2, however, showed that the amino acids formed in the bacteroids were not used as respiratory substrates in our case. Since the incubation system in Fig. 2 was bubbled with continuous O_2/Ar , the ^{14}C -amino acid formed would be due to endogenous NH_4^+ that was accumulated in the bacteroids and the concentration might be too small for the enzymes responsible for amino acid catabolism.

When ^{14}C accumulation in various organic acids was analyzed by HPLC, only two ^{14}C peaks were detected (Fig. 3), and they were suggested to be a polymer of sugar-acid (P-1) and succinate + malate (P-2). Since about 80% of the ^{14}C in P-2 was of succinate and the radioactivity of P-2 decreased during the incubation time with a constant ratio between succinate and malate, ^{14}C contents in organic

acids of the citric acid cycle except succinate and malate were suggested to be too low to calculate their specific radioactivity. Though the ^{14}C flow from exogenous succinate to CO_2 was vigorous and the organic acids pool sizes of citric acid cycle in the bacteroids did not change much during the incubation time (Table I), ^{14}C contents in major organic acids of citric acid cycle did not increased enough to be detected (less than 1% of ^{14}C content of succinate fraction). Only when the bacteroids were incubated with large amounts of ^{14}C -succinate continuously, incorporation of ^{14}C into 2-oxoglutarate and other organic acids was observed after the acids were separated by HPLC.¹⁷⁾ Our working conclusion was that the active pools of organic acids in the bacteroids were small and separated from large inactive ones. A relatively small ^{14}C -organic acid pool in the bacteroids was observed even when exogenous ^{14}C -succinate was supplied to the bacteroids continuously,¹⁷⁾ and the data also supported this conclusion. Similar result was obtained from the constant-ratio isotope experiment with intact soybean nodules by Kouchi *et al.*,⁶⁾ though the mechanism for the compartmentation of metabolic pools were obscure.

Though the pulse-chase experiment failed to indicate the succinate metabolizing pathway, no accumulation of ^{14}C -ethanol, ^{14}C -lactate or ^{14}C -pyruvate from [2,3- ^{14}C]succinate indicated negligible anaerobic metabolism in the bacteroids and suggested the operation of citric acid cycle (Fig. 3).

Further experiments to analyze the oxidation of exogenous succinate through the citric acid cycle in the bacteroids were done by a radio-respirometric technique. Figure 4a indicated that the methylene carbons of succinate were introduced into CO_2 much more slowly than carboxyl carbons, and Fig. 4b showed a similar result for acetate. Inhibitors of aconitase (fluoroacetate) and of succinate dehydrogenase (malonate) depressed $^{14}\text{CO}_2$ formation from [1,4- ^{14}C]succinate. All these data supported the oxidative degradation of succinate through citric acid cycle in the bac-

teroids even under 2% oxygen. Operation of glyoxylic acid cycle in the bacteroids was reported,¹⁴⁾ but Fig. 4b shows the contribution of glyoxylic acid cycle was minor though the bacteroids can form $^{14}\text{CO}_2$ from [1- ^{14}C]glyoxylate (data not shown).

HPLC analysis of the neutral fraction of the bacteroids strongly suggested that the ^{14}C in [2,3- ^{14}C] succinate was incorporated into a disaccharide. Elution profiles of the compound were not of trehalose, but of maltose. Maltose was already reported in the bacteroids though trehalose formation in the bacteroids was more extensive.^{12,13)}

When succinate oxidation in the bacteroids through citric acid cycle are vigorous, NADH and reductant generated must be reoxidized immediately for continuous operation of the cycle. Table II shows the presence of an active NADH-oxidizing system in the bacteroids since the endogenous NADH content decreased even after the addition of succinate to the incubation system. Nodule and root tissues showed higher NAD^+/NADH ratios (12 and 5.3 respectively,¹⁸⁾ and the rather low value of bacteroids were suggested to be a characteristic of bacteroid energy metabolism. The adenylate energy charge value in the bacteroids responded to succinate addition to the incubation system (Table 2). In 0.5 min the value increased from 0.25 to 0.53, then depressed to 0.29, followed by a gradual increase to 0.33 in 30 min. In this case, the incubation system contained no nitrogen and the nitrogenase system would be operating to generate hydrogen from water.

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