The Relationship between Nitrogenase and Glutamine Synthetase in Bacteroids of *Rhizobium leguminosarum* of Various Ages

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The activities of nitrogenase, glutamine synthetase and other ammonia-assimilating enzymes were studied in bacteroids of *Rhizobium leguminosarum* isolated 9 to 21 d after infection. Total bacteroid numbers (per plant) increased proportionally with nitrogenase, but glutamine synthetase activity decreased with increasing age of the root nodule. Glutamine synthetase was adenylated throughout the period of increasing nitrogenase activity and during the period of constant nitrogenase activity.

INTRODUCTION

Cultures of the genus *Rhizobium* have two systems for assimilating ammonia: ATP-independent glutamate dehydrogenase with low affinity for ammonia, and an ATP-dependent system with high affinity for ammonia which operates through the concerted action of glutamine synthetase and glutamate synthase (Brown & Dilworth, 1975). The presence of both systems is regulated by the exogenous ammonia concentration.

Leguminous plants become independent of supplied ammonia after infection with rhizobia and establishment of an effective symbiosis, in which the invading bacteria develop into non-dividing, ammonia-producing bacteroids. However, bacteroids of *Rhizobium leguminosarum* contain insufficient amounts of ammonia-assimilating enzymes to cope with all the newly fixed nitrogen (Kurz, Rokosh & La Rue, 1975; Brown & Dilworth, 1975; Planqué *et al.*, 1977). The excess ammonia is thought to be assimilated by an enzyme system consisting of glutamine synthetase, glutamate synthase and asparagine synthetase present in the plant fraction of root nodules (Scott, Farnden & Robertson, 1976; Robertson, Warburton & Farnden, 1975).

Glutamine synthetase has a dual role in some bacteria. In addition to its role in the production of glutamine from glutamate and ammonia, it controls the synthesis of some enzymes concerned with the breakdown of substrates used as nitrogen sources, e.g. histidase and proline oxidase (Magasanik, Prival & Brenchley, 1973). Nitrogenase synthesis is also considered to be under the control of glutamine synthetase (Shanmugam & Valentine, 1975). Mutants of *Klebsiella pneumoniae* with altered regulation of glutamine synthetase could not synthesize nitrogenase, and mutants of *K. aerogenes* constitutive for glutamine synthetase, which acquired the *nif* genes by genetic transfer, showed constitutive expression of these *nif* genes (Tubb, 1974). Results obtained for a *R. meliloti* mutant with low glutamine synthetase activity and a 'cow pea' *Rhizobium* lacking glutamine synthetase activity, both of which were deficient in nitrogenase, also indicate the involvement of glutamine synthetase in the regulation of nitrogenase synthesis (Kondorosi *et al.*, 1977; Ludwig & Signer, 1977).

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This paper describes studies of the activities of glutamine synthetase and nitrogenase during the transition of bacteria into bacteroids, using the techniques of Planqué & van Brussel (1976) to achieve simultaneous development of root nodules resulting in bacteroid preparations of reproducible age distribution.

METHODS

Bacteroids of *Rhizobium leguminosarum* strain A171 were produced by inoculation of pea seeds, *Pisum sativum* cv. Rondo, using growth conditions described previously (Planqué & van Brussel, 1976).

Bacteroids were isolated and purified as described by Planqué *et al.* (1977) except that 50 mM-Tris/HCl buffer, pH 7.4, was used instead of phosphate buffer. The purified bacteroids were resuspended in 50 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer, pH 7.4. Electron microscopic examination showed the final bacteroid preparation to be free from plastid and mitochondrial contamination (Planqué *et al.*, 1977).

Enzymic activities (excluding nitrogenase) were determined in cell-free homogenates prepared by sonicating the bacteroid suspension for 45 s and removing the debris by centrifuging at 50000 *g* for 45 min. The total bacteroid material isolated was determined by measuring the absorbance at 617 nm of a diluted sample of the bacteroid suspension prior to sonication, and converting this absorbance to bacteroid numbers using a calibration curve. Viable bacteria inside the root nodules were counted by the method of Vincent (1970). Duplicate samples of two root systems were sterilized for 3 min in 0.1 % (w/v) HgCl₂, and then homogenized in sterile water with an Elvehjem-Potter homogenizer. After removal of plant residue by filtration, appropriate dilutions were plated on mannitol/glucose/yeast extract agar (van Brussel, Planqué & Quispel, 1977).

Glutamine synthetase (EC 6.3.1.2) was determined by the transferase assay in the presence of Mn²⁺ (Shapiro & Stadtman, 1970). The average state of adenylation was determined by carrying out the transferase assay in the presence of either 0.3 mM-Mn²⁺ or 0.3 mM-Mn²⁺ plus 60 mM-Mg²⁺ and determining the ratio of the two values (Stadtman *et al.*, 1970).

Nitrogenase was assayed by acetylene reduction using 10 plants selected at random from a large culture. Roots were cut under the cotyledons and incubated for up to 20 min under 10 % (v/v) acetylene in air. Acetylene reduction was linear over this period. With the gas chromatographic procedures used (Planqué & van Brussel, 1976), an increase of 0.1 nmol ethylene could be detected.

Glutamate synthase (EC 2.6.1.53) and glutamate dehydrogenase (EC 1.4.1.4) were determined as described previously (Planqué *et al.*, 1977).

Protein was determined by a modified Folin procedure (Lowry *et al.*, 1951; Benzadoun & Weinstein, 1976).

RESULTS

Plant culture

As the plants were cultivated at an initial high level of medium in the growth-bin, root growth was inhibited, resulting in initiation of root nodules over a relative short piece of the main root (Planqué & van Brussel, 1976). At the proximal side of the meristem of *R. leguminosarum* root nodules, cells are infected by bacteria, resulting in an approximately constant number of young bacteroids. The amount of mature bacteroid tissue per plant increases up to 21 d after infection (Kijne, 1975*a, b*). This method of cultivation resulted in an almost synchronized development of the bacteroid tissue providing the experimenter with reproducible numbers of bacteroids on specific days after inoculation.

Development of bacteria and bacteroids in nodules

Preliminary experiments showed that surface sterilization of root nodules for up to 3 min resulted in a considerable decrease in the number of viable bacteria. Prolonged sterilization, for up to 20 min, had little further effect on the numbers of bacteria, but a period of 30 min resulted in complete sterilization of the nodule. This biphasic decrease in numbers of bacteria was taken to indicate that sterilization for up to 3 min killed organisms on the surface without significantly affecting the numbers of internal bacteria. We therefore sterilized the root nodules for 3 min before homogenization for measurements of the numbers of bacteria inside the nodules.

Fig. 1. Numbers of bacteria (x) and bacteroids (●) inside roots of *Pisum sativum*, and protein content (○) of sonic extract of bacteroids.

Fig. 2. Nitrogenase activity in plants of various ages; bars indicate the variation between samples of the same age. Activities (per plant) are expressed as $\mu\text{mol acetylene reduced h}^{-1}$.

Fig. 3. Specific activity of glutamine synthetase in bacteroid cytoplasm, determined as Mn^{2+} -activated γ -glutamyl transferase. Activities are expressed as $\text{nmol } \gamma\text{-glutamyl hydroxamate formed (mg protein)}^{-1} \text{ min}^{-1}$.

Numbers of bacteria increased from 0.4×10^6 at day 9 to 4×10^6 at day 16, while bacteroid numbers increased from 2.4×10^9 per plant at day 9 to 30.2×10^9 at day 20 (Fig. 1). Bacterial numbers never exceeded 0.02% of the number of bacteroids. The increase in bacteroid numbers was accompanied by a linear increase in soluble protein in the supernatant of disrupted bacteroids (Fig. 1).

Development of enzymic activities

Nitrogenase was first detected 9 d after infection. Initially the increase in activity was low, but between days 11 and 16 a rapid increase was observed (Fig. 2). The steady state activity at day 20 was 2.5 to 3.5 $\mu\text{mol acetylene reduced per plant h}^{-1}$.

Glutamine synthetase showed a notable variation between bacteroids of the same age isolated from different plant cultures. Attempts to minimize the variation by optimizing the assay conditions were unsuccessful. Despite this variation, we concluded from Fig. 3 that specific activities were higher in young bacteroids (9 to 12 d) and that activity was negligible after day 16. In previous experiments, we reported the absence of biosynthetic activity in bacteroids isolated at day 21, using the synthetase assay (Planqué *et al.*, 1977).

Fig. 4. Relation between nitrogenase and glutamine synthetase specific activities. Nitrogenase specific activities [$\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$] were determined by dividing the nitrogenase activity of intact plants by the protein concentration of bacteroid cytoplasm of plants of the same culture and age.

As a further control on the adenylation state of glutamine synthetase, we assayed the enzyme in the presence of either 0.3 mM-Mn^{2+} or 0.3 mM-Mn^{2+} plus 60 mM-Mg^{2+} at days 10, 14 and 21. The Mn^{2+} plus Mg^{2+} activated assays showed no significant activity at all ages of the bacteroids.

There was a reciprocal relationship between the specific activities of nitrogenase [converted from acetylene reduction data as described by Planqué *et al.* (1977)] and glutamine synthetase (Fig. 4). Where acetylene reduction and glutamine synthetase activities were determined on the same samples, much less variation was observed than one would expect from Fig. 3. Glutamate synthase was absent from all samples investigated.

Glutamate dehydrogenase activity was always detected. The total activity, expressed on a per plant basis, showed a small increase from 0.1 to $1.0 \text{ nmol NADH oxidized min}^{-1}$ between days 9 and 14 after which it fluctuated between 2 and $6.5 \text{ nmol NADH oxidized min}^{-1}$. The specific activity decreased. With the exception of the sample on day 9, the specific activities were inadequate to account for the assimilation of newly fixed nitrogen.

DISCUSSION

The co-development of total bacteroid numbers, bacteroid protein and nitrogenase during maturation of bacteroid tissue concurs with the anatomical evidence that development of bacteroid tissue in this *Pisum sativum*–*Rhizobium leguminosarum* symbiosis, under greenhouse conditions, continues until breakdown of the oldest bacteroid tissue starts after about 21 d (Kijne, 1975*b*). In a similar experiment on age dependency of nitrogenase and ammonia-assimilating enzymes, Kurz *et al.* (1975) also found a time-dependent relationship between these enzymes. However, their experiments were done under field conditions over a 13-week period and in this system secondary infections on lateral roots would disturb the general pattern of development in bacteroid tissue, resulting in a less clear relationship. The experiments presented here show a strict relationship between the activity of glutamine synthetase and nitrogenase and the age of bacteroid tissue in this symbiotic association.

Glutamine synthetase and glutamate dehydrogenase lose specific activity during the increase in bacteroid numbers. It is unlikely that either enzyme plays a significant assimilatory role. Glutamate dehydrogenase has too high an apparent K_m for ammonia (Dunn & Klucas, 1973; Brown & Dilworth, 1975) and glutamine synthetase is present in a catalytically inactive form. The glutamine synthetase of *R. leguminosarum* is thus different from that of

R. japonicum bacteroids and 'cow pea' *Rhizobium* in that the enzymes from the latter two species have low adenylylation numbers (Bishop *et al.*, 1976; Bergersen & Turner, 1976) in, respectively, active nitrogen-fixing bacteroids and free-living nitrogen-fixing bacteria, whereas this study and that of Kurz *et al.* (1975) has shown that glutamine synthetase in *R. leguminosarum* is highly adenylylated. Increasing the ammonia concentration, whether applied to intact nodules or to isolated bacteroids, did not affect the degree of adenylylation in *R. japonicum* bacteroids, although it did affect the nitrogenase activity suggesting that the adenylylation cascade system does not function in the bacteroids (Bishop *et al.*, 1976). In the free-living nitrogen-fixing 'cow pea' *Rhizobium* grown in continuous culture, excess ammonia also had no effect on the degree of adenylylation provided that stringent oxygen limitation was maintained. Increasing oxygen concentration, however, resulted in inactivation of the glutamine synthetase by adenylylation (Bergersen & Turner, 1976). These bacteria are supposedly dividing in contrast to the bacteroids of root nodules. Ludwig & Signer (1977) demonstrated that in free-living 'cow pea' *Rhizobium*, nitrogenase activity did not appear before the cells had reached the stationary phase of growth and therefore the nitrogenase activity shown in the continuous culture experiments of Bergersen & Turner (1976) could be attributed to a non-dividing population of cells.

The results presented here show inactive glutamine synthetase at all ages of bacteroids investigated.

The validity of the transferase reaction for the assay of glutamine synthetase has been doubted (Mifflin & Lea, 1977; Darrow, Knotts & Jarrel, 1976) and extrapolation of the assay conditions for *E. coli* to *Rhizobium* may not result in optimal determination of the state of adenylylation in *Rhizobium*. However, glutamine synthetase activity was also not detected using the synthetase assay in pea bacteroids (Planqué *et al.*, 1977).

Kondorosi *et al.* (1977) found no evidence for the functioning of an adenylylation cascade system in *Rhizobium meliloti* in batch culture experiments. Changes in the state of adenylylation in intact cells, which could be prevented by the use of cetyltrimethylammonium bromide (Bender *et al.*, 1977), have been observed in *R. leguminosarum*, indicating the presence of an active adenylylating system (de Vries & Schouten, unpublished results).

The disappearance of glutamine synthetase from the bacteroids is probably due to the absence of synthesis of the enzyme during and after the transition of bacteria to bacteroids.

The hypothesis that in *Rhizobium* symbiosis the de-adenylylated glutamine synthetase has to be present to derepress nitrogenase synthesis is not supported by the results presented here. Possibly de-adenylylated glutamine synthetase is present in very young bacteroids sufficient to derepress nitrogenase synthesis during the active life span of the bacteroid but in an amount which escapes detection in the assay.

The dependence of glutamine synthetase activity on bacteroid age should be taken into account in future studies of this enzyme in bacteroids.

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