Asparagine degradation in *Rhizobium etli*

Alejandra Huerta-Zepeda, Socorro Durán, Gisela Du Pont and Jorge Calderón

Author for correspondence: Jorge Calderón. Tel: +52 6 22 38 80. Fax: +52 5 50 00 48.

The degradation of asparagine by *Rhizobium etli* involves asparaginase and aspartate ammonia-lyase (L-aspartase). The two enzymes were shown to be positively regulated by asparagine and negatively regulated by the carbon source. Asparaginase activity was not regulated by oxygen concentration or by nitrogen catabolite repression. Induction of both enzymes by asparagine enables *R. etli* to utilize asparagine as carbon source. Asparaginase may also be involved in maintaining the optimal balance between asparagine and aspartate. Aspartase was not involved in the utilization of aspartate or glutamate as carbon source. The presence of high levels of the two enzymes in *R. etli* bacteroids suggests that they may have a role in symbiosis between *R. etli* and *Phaseolus vulgaris*.

Keywords: *Rhizobium etli*, asparaginase, aspartase, catabolism, symbiosis

INTRODUCTION

In some organisms, L-asparagine can be utilized as sole carbon and nitrogen source through the action of two enzymes (Sun & Setlow, 1991). The first enzyme, asparaginase (EC 3.5.1.1), catalyses the hydrolysis of L-asparagine to L-aspartate and ammonium. The second enzyme, aspartase ammonia-lyase (L-aspartase; EC 4.3.1.1), catalyses the reversible deamination of L-aspartate to yield fumarate and ammonium.

Asparaginase activity has been studied in *Escherichia coli* and other Gram-negative bacteria such as *Salmonella enterica* (Jennings et al., 1993), *Erwinia chrysanthemi* (Gilbert et al., 1986) and *Vibrio proteus* (Sinha et al., 1991), and in Gram-positive bacteria such as *Bacillus subtilis* (Sun & Setlow, 1991) and *Staphylococcus aureus* (Sobis & Mikucki, 1991; Rózalska & Mikucki, 1992). *E. coli* contains two L-asparaginase isoforms. L-Asparaginase I has a low affinity for asparagine, it is cytoplasmic and is thought to be constitutively produced (Jerlström et al., 1989; Willis & Woolfolk, 1974); L-asparaginase II is a high-affinity enzyme secreted to the periplasm, and its expression is positively regulated by the cAMP receptor protein and by anaerobiosis via the product of the *fur* gene (Cedar & Schwartz, 1967; Chesney, 1983; Ho et al., 1970; Jerlström et al., 1987; Russell & Yamasaki, 1978; Jennings & Beachman, 1993).

In *S. enterica*, an organism closely related to *E. coli*, L-asparaginase II is regulated by glucose levels in the medium, via the catabolite repressor protein, and by anaerobiosis. However, the anaerobic regulation of this gene does not act via the FNR protein, contrasting with the case in *E. coli* (Jennings et al., 1993). *B. subtilis* L-asparaginase and L-aspartase are encoded by the *ans* operon, which is subject to strong repression by a good nitrogen source, such as ammonium, and is also induced by asparagine and aspartate (Sun & Setlow, 1991; Atkinson & Fisher, 1991; Iijima et al., 1977).

L-Aspartase activity has been studied in several bacteria, such as *E. coli*, *B. subtilis* (Sun & Setlow, 1991), *Pseudomonas fluorescens* (Tokushige, 1985; Miyamoto & Katsuki, 1992) and *Serratia marcescens* (Takagi & Kisumi, 1985). *E. coli* aspartase is activated by divalent ions (Rudolph & Fromm, 1971; Karsten & Viola, 1991) and regulated by catabolite repression and by anaerobiosis via the product of the *fur* gene (Nishimura & Kisumi, 1984; Jerlström et al., 1987).

Bacteria of the genus *Rhizobium* establish intracellular symbiosis with legumes in which the bacteroids fix atmospheric nitrogen and export ammonium to plant tissues. Carbon compounds produced by the plant are metabolized by the bacteria to supply the energy and reductant needed for nitrogen fixation. Identification and analysis of the bacterial and plant enzymes that are active in the nodule are important to understand the metabolic pathways used by both partners during symbiosis.

Kahn et al. (1985) proposed that the carbon supplied to bacteroids includes amino acids. Rastogi & Watson (1991) found that a *Rhizobium meliloti* mutant, unable to grow on aspartate as carbon source due to a deficiency of a novel atypical aspartate aminotransferase activity, does not fix...
nitrogen, implying that aspartate is an essential substrate for bacteroids in the nodule (Watson & Rastogi, 1993).

Considering the importance that amino acid metabolism may have in the Rhizobium symbiosis, together with the finding that Rhizobium etli does not grow on aspartate but does grow on asparagine as carbon source, and that high asparaginase and aspartase activities have been found in Rhizobium lupini bacteroids (Kretovich et al., 1981), we decided to study the degradation of asparagine in R. etli, a bacterium that establishes symbiosis with Phaseolus vulgaris (bean) (Segovia et al., 1993).

In this report, we present evidence of an asparaginase–aspartase pathway for the degradation of asparagine in R. etli and the regulation of these enzymes. The enzymic activities of asparaginase and aspartase in bacteroids are also reported.

**METHODS**

**Strains.** The Rhizobium etli CNF42 wild-type strain used in this study was previously classified as Rhizobium leguminosarum biovar phaseoli (Segovia et al., 1993).

**Growth conditions.** Batch cultures of R. etli were grown at 30 °C with shaking at 200 r.p.m. For growth on minimal medium (MM) (Beringer, 1974), cells previously grown overnight on a rich medium (PY) containing 0.5% (w/v) peptone, 0.5% (w/v) yeast extract and 7 mM CaCl₂ were washed and used as inoculum. The initial OD₅₆₀ of the medium was adjusted to 0.05. The nitrogen and carbon sources in MM were added at 10 mM, except for glycerol (2%, v/v).

**Microaerobic cultures were achieved by flushing a tightly stoppered Erlenmeyer flask containing 250 ml medium with nitrogen for 10 min and re-admitting air to give a 1% (v/v) oxygen concentration. Growth was monitored by measurement of the OD₅₆₀ and by protein determination using the Lowry method.

**Determination of asparaginase activity.** Cell extracts were prepared by sonicating whole cells in extraction buffer (100 mM KH₂PO₄, pH 7.6) with a Soniprep 150 ultrasonic disintegrator; homogenates were centrifuged for 5 min at 12000 r.p.m. (Beckman JA-20 fixed-angle rotor) at 4 °C. The assay measuring ammonium formation was performed in a 0.25 ml reaction mixture containing 30 mM KH₂PO₄, pH 7.6, 10 mM asparagine and 0.05 ml cell extract. Ammonium formation was measured as described elsewhere (Chaney & Marbach, 1962; Durán & Calderón, 1995). The assay measuring the formation of aspartate was performed in a 1 ml reaction mixture containing 30 mM KH₂PO₄, pH 7.6, 10 mM asparagine and 0.2 ml cell extract. Aspartate was determined with a System Gold Liquid Chromatographic system from Beckman and a Gilson Fluorometer model 121 after coupling with o-phthalaldehyde (Calderón et al., 1985).

**Determination of glutamine synthetase activity.** Glutamine synthetase activity was assayed with cell extracts prepared by sonicating the cells in 10 mM imidazole hydrochloride and 0.5 mM EDTA (pH 7.0). It was measured by its synthetase activity as described by Bender et al. (1977).

**Bacteroid isolation.** Bacteroids from root nodules of P. vulgaris were isolated by utilizing self-generating Percoll gradients as described by Reibach et al. (1981). A crude extract was prepared by gently crushing 6 g nodules with a mortar and pestle in 15 ml grinding buffer (150 mM NaCl and 50 mM KH₂PO₄, pH 7.6). The crude homogenate was filtered through four layers of cheesecloth and the residue was washed with additional grinding buffer. The filtered crude extract was centrifuged for 5 min at 3000 r.p.m. (Beckman Instrument JA-20 fixed-angle rotor) at 4 °C, the supernatant was centrifuged again for 15 min at 15000 r.p.m. and the pellet was resuspended in 2 ml grinding buffer. A 50 ml polycarbonate centrifuge tube was filled with 24.5 ml Percoll concentrate, 3.5 ml 500 mM KH₂PO₄ plus 1.5 M NaCl, pH 7.6, and 7 ml H₂O₂ giving a final concentration of 70% Percoll. One millilitre of extract was layered on the top of the gradient. The tube was centrifuged for 50 min at 20000 r.p.m. The bacteroids were collected with a Pasteur pipette. Percoll was removed by diluting the fraction 1:10 with 150 mM NaCl and 50 mM KH₂PO₄, pH 7.6, and centrifuging for 20 min at 15000 r.p.m. The pellet was resuspended in 2 ml extraction buffer.

**RESULTS AND DISCUSSION**

**Growth of R. etli on aspartate and asparagine as nitrogen and carbon source**

R. etli grew very poorly on aspartate as nitrogen and carbon source, whereas on asparagine as nitrogen source and succinate as carbon source it grew as well as on ammonium plus succinate (Fig. 1). In contrast, on glutamate as nitrogen and carbon source, R. etli grew at half the rate observed with glutamate plus succinate. On asparagine as nitrogen and carbon source, R. etli grew almost as well as on asparagine plus succinate (Fig. 1). These results indicate that R. etli utilized aspartate, glutamate and asparagine as nitrogen sources, that aspartate is a poor carbon source and that asparagine is a good carbon source, in spite of the fact that in order to be utilized as carbon source, asparagine must first be degraded to aspartate.
Asparagine degradation in *Rhizobium etli*

**Asparaginase**

The assay conditions for measurement of asparaginase activity were optimized. It was shown that the amounts of aspartate (1.05 pmol) and ammonium (1.16 pmol) formed were similar.

Asparaginase activity was highest when *R. etli* was grown on asparagine as nitrogen and carbon source, and this activity decreased when *R. etli* was grown on asparagine plus another carbon source such as glycerol, glucose or succinate (Table 1). Asparaginase activity was similar when *R. etli* was grown on asparagine plus succinate in the absence or presence of ammonium, and low when bacteria were grown on a good inorganic nitrogen source such as ammonium or a poor nitrogen source such as nitrate. Asparaginase activity was low in cells grown on aspartate with or without succinate such as in ammonium plus succinate. When *R. etli* was grown on other amino acids such as glutamine, and glutamate with or without succinate, or on a rich PY medium, asparaginase activity was low (Table 1). When *R. etli* was grown on ammonium plus succinate, asparaginase activity was similar during the different growth stages (data not shown). The amount of oxygen dissolved in the growth medium was found to be irrelevant to the asparaginase activity (Table 1).

This shows that asparaginase was: (a) positively regulated by its substrate asparagine as in *B. subtilis* (Sun & Setlow, 1991) and *V. proteus* (Sinha *et al.*, 1991), and (b) negatively regulated by the carbon source as shown by the reduction in the asparaginase activity when a carbon source such as glycerol, glucose or succinate was present in the growth medium in addition to asparagine. Similar findings have been reported for *E. coli* (Jennings & Beachman, 1993), *S. enterica* (Jennings *et al.*, 1993) and *Staph. aureus* (Rózalska & Mikucki, 1992).

*R. etli* asparaginase was not regulated by: (a) the amount of oxygen dissolved in the growth medium, in contrast to other bacteria such as *E. coli* (Jennings & Beachman, 1993), *S. enterica* (Jennings *et al.*, 1993) and *V. proteus* (Sinha *et al.*, 1991); (b) nitrogen catabolite repression, as shown by the inability of ammonium to reduce asparaginase activity and the low activity found when *R. etli* was grown on a poor nitrogen source (Table 1), in contrast with data for *B. subtilis* (Sun & Setlow, 1991), *Bacillus licheniformis* (Golden & Bernlohr, 1985) and *Saccharomyces cerevisiae* (Roon *et al.*, 1982). Our results suggest that *R. etli* asparaginase plays a catabolic role in the degradation of asparagine to carbon skeletons when this amino acid is present in the growth medium and may also play a role in maintaining the balance between asparagine and aspartate since some activity is present when *R. etli* is grown in medium without asparagine.

**Aspartase**

We found that magnesium was necessary in the assay mixture for optimal *R. etli* aspartase activity, giving a threefold stimulation. In the complete assay, the amounts of ammonium (1.14 µmol) and fumarate (1.07 µmol) formed were similar.

The influence of nutritional conditions on aspartase activity was examined. The highest aspartase activity was found when *R. etli* was grown on asparagine as nitrogen and carbon source, suggesting positive regulation as in *B. subtilis* (Sun & Setlow, 1991). This activity decreased when *R. etli* was grown on asparagine plus another carbon source such as glycerol, glucose or succinate (Table 1), suggesting negative regulation as in *E. coli* (Nishimura & Kisumi, 1984). Aspartase activity decreased when *R. etli* was grown on asparagine plus succinate in the presence of ammonium, suggesting repression by ammonium. Aspartase activity was low when bacteria were grown on a good inorganic nitrogen source, such as ammonium, or on a poor nitrogen source, such as nitrate. Aspartase activity was also low in cells grown on aspartate with or without succinate. When *R. etli* was grown on other amino acids such as glutamine, and glutamate with or without succinate, or on a rich PY medium, aspartase activity was low (Table 1). The amount of oxygen dissolved in the growth medium was found to be
irrelevant to the aspartase activity (Table 1), in contrast with other bacteria such as E. coli.

Aspartate aminotransferase, fumarase and glutamine synthetase

The activity of aspartate aminotransferase, an enzyme involved in aspartate catabolism, was determined in R. etli crude extracts. This enzyme catalyses the reversible conversion of aspartate plus 2-oxoglutarate to glutamate plus oxaloacetate. We found that the levels of the enzyme were similar [310 nmol min\(^{-1}\) (mg protein\(^{-1}\))\] when R. etli was grown on ammonium plus succinate, PY medium and asparagine or aspartate with or without succinate.

The activity of the Krebs cycle enzyme fumarate hydratase (fumarase), which catalyses the reversible conversion of fumarate to malate, was determined. The levels of this enzyme were similar when R. etli was grown on asparagine or aspartate plus succinate [550 nmol min\(^{-1}\) (mg protein\(^{-1}\))\]. This activity was slightly lower when R. etli was grown on asparagine or aspartate [390 nmol min\(^{-1}\) (mg protein\(^{-1}\))\] higher than with ammonium plus succinate. The levels of this enzyme under conditions of asparagine and aspartate were similar (sevenfold lower than on ammonium plus succinate).

The fact that R. etli does not grow on aspartate as nitrogen and carbon source despite containing aspartase is explained by the very low aspartase activity found under these conditions. The poor growth on aspartate is not due to lack of fumarase or glutamine synthetase since these enzymes activities were similar when R. etli was grown on asparagine or aspartate as nitrogen and carbon source. The higher glutamine synthetase activity found when grown on aspartate plus succinate, in comparison with asparagine plus succinate, indicates that aspartate is a poor nitrogen source, since glutamine synthetase is induced under conditions of nitrogen limitation (Bravo & Mora, 1988). The poor growth on aspartate as nitrogen and carbon source indicates an incapacity of enzymes otherwise capable of degrading aspartate under these conditions, such as aspartate aminotransferase, the enzyme responsible for the growth of R. meliloti under the same conditions (Rastogi & Watson, 1991; Watson & Rastogi, 1993). The low aspartase activity found on glutamate as nitrogen and carbon source indicates that glutamate must be degraded to carbon skeletons by another enzyme. It is interesting that the physiological role of aspartase is not to enable R. etli to utilize aspartate or glutamate as carbon source. Instead, aspartase is induced by asparagine along with asparaginase to enable R. etli to utilize asparagine as carbon source. This contrasts with B. subtilis, where

<table>
<thead>
<tr>
<th>Nitrogen/carbon source</th>
<th>Asparaginase Specific activity [nmol ammonium min(^{-1}) (mg protein(^{-1}))]</th>
<th>Aspartase Specific activity [nmol fumarate min(^{-1}) (mg protein(^{-1}))]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine</td>
<td>34.6 (6)</td>
<td>390.3 (50)</td>
</tr>
<tr>
<td>Asparagine/glycerol</td>
<td>14.0 (2)</td>
<td>32.3 (6)</td>
</tr>
<tr>
<td>Asparagine/glucose</td>
<td>12.2 (2)</td>
<td>26.8 (4)</td>
</tr>
<tr>
<td>Asparagine/succinate</td>
<td>16.0 (3)</td>
<td>261.0 (30)</td>
</tr>
<tr>
<td>Asparagine/ammonium/succinate</td>
<td>13.7 (3)</td>
<td>924.15 (23.7)</td>
</tr>
<tr>
<td>Ammonium/glycerol</td>
<td>3.7 (1)</td>
<td>84.2 (2)</td>
</tr>
<tr>
<td>Ammonium/glucose</td>
<td>6.0 (1)</td>
<td>1.5 (1)</td>
</tr>
<tr>
<td>Ammonium/succinate</td>
<td>4.5 (1)</td>
<td>16.2 (3)</td>
</tr>
<tr>
<td>Nitrate/succinate</td>
<td>5.5 (1)</td>
<td>10.0 (2)</td>
</tr>
<tr>
<td>Asparate/succinate</td>
<td>4.1 (1)</td>
<td>8.1 (2)</td>
</tr>
<tr>
<td>Glutamine/succinate</td>
<td>2.5 (1)</td>
<td>12.0 (2)</td>
</tr>
<tr>
<td>Glutamate/succinate</td>
<td>3.5 (1)</td>
<td>7.8 (2)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>4.7 (1)</td>
<td>30.4 (7)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.7 (1)</td>
<td>12.1 (2)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3.4 (1)</td>
<td>16.3 (3)</td>
</tr>
<tr>
<td>Peptone-yeast extract</td>
<td>2.9 (1)</td>
<td>3.2 (1)</td>
</tr>
<tr>
<td>Asparagine/succinate, low O(_2)</td>
<td>15.3 (2)</td>
<td>230.5 (25)</td>
</tr>
<tr>
<td>Ammonium/succinate, low O(_2)</td>
<td>4.7 (1)</td>
<td>15.2 (3)</td>
</tr>
</tbody>
</table>

Table 1. Asparaginase and aspartase activities of R. etli grown under different conditions

The activities were measured after 18 h growth. The numbers in parentheses represent the standard deviations.
Asparaginase and aspartase activities of bacteroids from *R. etli*

The role of asparagine-degrading enzymes in bacteroids was analysed by isolating *R. etli* bacteroids in a self-generating Percoll gradient (Reibach et al., 1981) and assessing the asparagine-degrading enzyme activities. The nodules were harvested 28 d post-inoculation. The asparaginase activity in bacteroids [38 nmol min⁻¹ (mg protein)⁻¹] was as high as the activity found when *R. etli* was grown on asparagine as nitrogen and carbon source (Table 1). The aspartase activity was also high in bacteroids [164 nmol min⁻¹ (mg protein)⁻¹].

These observations may indicate roles for these two enzymes in the symbiosis between *R. etli* and *P. vulgaris*. The degradation of asparagine by asparaginase and aspartase in bacteroids may also be important in other species of *Rhizobium*, such as *R. lupini*, where levels of these enzymes have been found to be elevated (Kretovich et al., 1981).

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REFERENCES


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