Ammonia Regulation of Glutamine Synthetase in *Rhizobium* sp. ANU289

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*Rhizobium* sp. ANU289 lacked glutamate dehydrogenase (GDH) but grew rapidly on ammonia in a well-buffered medium. The properties of glutamine synthetase I (GSI) differed from those of GS from enteric bacteria in a manner which appeared to compensate for the lack of GDH. Adenylylated and unadenylylated GSI had the same biosynthetic activity, and there was also no detectable difference between the two forms with respect to affinities for substrates. GSII, but not GSI, was repressed by growth on ammonia, further suggesting that GSI is important in ammonia assimilation in the presence of high ammonia concentrations. Methylamine was found to exert similar regulatory effects to ammonia and was therefore used to determine the extent to which utilization of various nitrogen sources was controlled by ammonia in strain ANU289.

**INTRODUCTION**

Two main pathways of ammonia assimilation exist in bacteria – via glutamate dehydrogenase (GDH; EC 1.4.1.3) (reaction 1) and via glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.13) (reactions 2 and 3). The end product of both pathways is glutamate which then acts as an amino group donor in various amino transferase reactions. GDH has a high $K_m$ for ammonia and is thus only operative at high ammonia concentrations. GS/GOGAT is the major route of ammonia assimilation when ammonia is limiting.

$$\text{NH}_4^+ + 2\text{oxoglutarate} + \text{NAD(P)H} \xrightarrow{\text{GDH}} \text{glutamate} + \text{NAD(P)H}$$

(1)

$$\text{NH}_4^+ + \text{glutamate} + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + \text{P}_i$$

(2)

$$\text{Glutamine} + 2\text{oxoglutarate} + \text{NADPH} \xrightarrow{\text{GOGAT}} 2\text{glutamate} + \text{NADP}^+$$

(3)

In enteric bacteria, the use of these two pathways is genetically controlled in response to the availability of ammonia. These enzymes form part of a general nitrogen control system (reviewed by Magasanik, 1982) which includes a number of enzymes involved in the breakdown of nitrogenous compounds. Ammonia is the preferred nitrogen source for enteric bacteria so when ammonia is present at a high concentration the genes for GS and other enzymes under nitrogen control are repressed while GDH is induced. The converse occurs when ammonia is limiting, giving the cell the capability to use a variety of nitrogen sources. Additionally, GS activity is controlled biochemically in response to ammonia. In the presence of ammonia, adenyl groups are reversibly attached to GS, causing inactivation. In the presence of high concentrations of ammonia, therefore, GS is both inactivated and its synthesis repressed so ammonia assimilation must occur through GDH.

Ammonia assimilation by *Rhizobium* differs from ammonia assimilation by enteric bacteria in a number of respects. *Rhizobium* uses ammonia for growth in the free-living state, but in the nitrogen-fixing bacteroid form all ammonia is exported to the plant fraction of the nodule.

**Abbreviations:** GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MA, methylamine; SVPD, snake venom phosphodiesterase.
glutamine synthetases: GSI, which is analogous in structure to the GS regulated by adenylylation, and GSII, which is the product of a separate gene (Darrow et al., 1981; Somerville & Kahn, 1983). Finally, some Rhizobium species lack a GDH so GS/GOGAT is the sole route of ammonia assimilation (Brown & Dilworth, 1975). Thus, even in free-living rhizobia, the route of ammonia assimilation differs from that used by enteric bacteria.

It is not known if Rhizobium spp. possess a co-ordinated nitrogen control system similar to that of the enteric bacteria. Studies with mutants of Rhizobium sp. 32H1 have suggested that GSI or deadenylating proteins are involved in nitrogenase expression but GSII appears to be regulated separately (Ludwig 1980). Further work needs to be done to determine whether this is part of a general nitrogen control system.

Rhizobium sp. ANU289 is a slow-growing strain which is capable of forming effective nodules on some tropical legumes, as well as on the non-legume Parasponia. In this study we show that strain ANU289 lacks GDH, and that the regulation of GS differs from that in enteric bacteria in such a way as to compensate for the absence of GDH. We also present the results of a preliminary investigation into the possibility that a co-ordinated nitrogen control system exists in this Rhizobium strain.

METHODS

Bacterial strain. Rhizobium sp. strain ANU289 is a non-mucoid derivative of strain CP283 (Trinick & Galbraith, 1980), which has been made resistant to 500 mg streptomycin l⁻¹ (Mohapatra et al., 1983).

Media and growth conditions. The basic (nitrogen-free) medium was N⁻FGM, which contained per litre: 150 mg NaH₂PO₄, 500 mg KCl, 250 mg Na₂SO₄, 7H₂O, 150 mg CaCl₂, 2·8 mg FeSO₄, 7H₂O, 3·7 mg Na₂EDTA, 10 mg inositol, 0·1 mg nicotinic acid, 0·1 mg pyridoxin. HCl, 0·001 mg thiamin. HCl, 0·001 mg biotin and 1 ml trace element stock (Gresshoff & Doy, 1974). The carbon source was gluconate (5 g l⁻¹) and 50 mM-MOPS, pH 6·8, was also included. Nitrogen sources and methylamine were filter sterilized and added to autoclaved N⁻FGM. All nitrogen sources were 10 mM unless stated otherwise. Cultures were grown at 30 °C and were agitated by shaking.

Preparation of cell-free extracts. Cells were collected by centrifugation at 13 000 g at 4 °C for 10 min, washed in 50 mM-MOPS, pH 7·0, containing 1 mM-MnCl₂ and finally resuspended in the same buffer. Cells were disrupted by sonication, and cell debris was removed by centrifugation at 37 000 g at 4 °C for 15 min. The crude extracts were then used for GS and amidase assays.

Enzyme assays. Two assays for GS activity were used: the biosynthetic assay and the γ-glutamyl transferase assay. The biosynthetic activity was determined by the method of Bender et al. (1977), except that cetyltrimethylammonium bromide was omitted from the assay solution. The transferase assay method described by Fuchs & Keister (1980a) was used, except that 0·85 mM-alanine was added to the assay solution. In the absence of alanine, no isoactivity point was observed because unadenylylated GSI had higher activity than adenylylated GSI at all pH values. Alanine inhibited unadenylylated GSI more than the adenylylated form so an isoactivity point existed at pH 8·0. The isoactivity point is the pH at which the activities of adenylylated and unadenylylated GSI are equal. It can therefore be used to determine GSI activity independently of adenylylation state.

GS and GSII activities were distinguished by using the heat lability of GSII. Incubation of the cell extracts at 60 °C for 15 min has been used to inactivate GSII in R. japonicum (Fuchs & Keister, 1980b), but at this temperature GSI of strain ANU289 was also slightly unstable so a 2 h treatment at 50 °C (Darrow & Knotts, 1977) was used. GSI was not affected at this temperature.

Amidase activity was determined by measuring the transferase activity (Brammar & Clarke, 1964). Cell extract (0·25 ml) was added to 0·25 ml 2·0 M-hydroxylamine. HCl, freshly neutralized, and 0·25 ml 50 mM-MOPS, pH 7·2. This mixture was allowed to equilibrate at 30 °C for 5 min. The assay was started by the addition of 0·25 ml 0·4 M-acetamide. After 15 min incubation at 30 °C the reaction was stopped by the addition of 2 ml stop mix, as used for the GS transferase assay (Fuchs & Keister, 1980a). The absorbance at 540 nm was read and compared with that of a standard of acetohydroxamic acid.

To ensure that the transferase assay was actually measuring the activity of the amidase, preliminary experiments were also done to measure the hydrolytic activity of the amidase. Cell extract was incubated at 30 °C with 0·4 M-acetamide and 50 mM-MOPS, pH 7·2, and the ammonia released was measured by the phenol/hypochlorite method (Gresshoff, 1981). The ratio of the hydrolytic to transferase activity remained constant over a wide range of growth conditions, which suggested that the transferase activity was due to an amidase.
Nitrate reductase activity was determined with whole cells; the method used was based on that described by Manhart & Wong (1979). Cells were collected by centrifugation at 4°C at 13000 g for 10 min, washed in 50 mM-KH₂PO₄ buffer, pH 7.2, and resuspended in the same buffer. The cells were then incubated at 30°C for 1 h with 40 mM-KNO₃, 20 mM-sodium succinate, 20 mM-glucose, 100 mg chloramphenicol l⁻¹ and 50 mM-KH₂PO₄, pH 7.2. The reaction was stopped by the addition of 0.5 ml 1 M-zinc acetate and 1.5 ml ethanol. Cell debris was removed by centrifugation and the amount of nitrite produced was then determined by the method of Nicholas & Nason (1957).

A modified Lowry procedure, described by Schacterle & Pollack (1973), was used to determine protein values.

Snake venom phosphodiesterase (SVPD) treatment. SVPD type VII was obtained from Sigma. It was made up in 0.1 mM-Tris/HCl, pH 9.0, containing 1 mM-MgCl₂. To deadenylylate GSI, 0.1 U SVPD was incubated at pH 9.0 with cell extract containing about 3 mg protein at 37°C for 30 min.

RESULTS

Pathways of ammonia assimilation

In a well-buffered medium (containing 50 mM-MOPS or HEPES) strain ANU289 was capable of rapid growth on ammonia, the growth rate being similar at ammonia concentrations from 5 to 50 mM. Thus strain ANU289 is able to tolerate and use ammonia at quite high concentrations. The GS/GOGAT pathway appeared to be the sole route of ammonia assimilation by strain ANU289. No GDH activity could be measured in cells grown at various ammonia concentrations. Thus the GS/GOGAT pathway must have sufficient activity to allow the use of ammonia at rates sufficient to give the observed growth rate.

Biochemical control of GS activity

Adenylylation of GS in enteric bacteria occurs rapidly in response to the addition of ammonia. When ammonia was added to cultures of strain ANU289 grown with glutamate as the sole nitrogen source, a change in the properties of GSI was observed. In some respects, this change resembled those observed following adenylylation of GS in other bacteria. However, ammonia shock in strain ANU289 did not result in increased sensitivity of GSI to inhibition by Mg²⁺, as it does in most other bacteria. To confirm that we were actually looking at a change in adenylylation, we examined the effect of SVPD, which catalyses deadenylylation (Tronick et al., 1973) on GSI from ammonia-shocked cells.

Table 1 shows the activity of GSI from untreated and ammonia-shocked cells, and ammonia-shocked cells treated with SVPD. To distinguish between different adenylylation states, four different sets of assay conditions were used: (a) the isoactivity point conditions (i.e. pH 8 with 0.85 mM-alanine included in the assay solution); (b) isoactivity point conditions plus 60 mM-MgCl₂; (c) pH 7 with 0.85 mM-alanine, where GSI from treated cells had higher activity than GSI from untreated cells, and (d) pH 8 with no alanine, where GSI from untreated cells had the higher activity. In the two cases where activity did change in response to ammonia shock the original activity was restored by SVPD treatment (Table 1), suggesting that the ammonia shock

<table>
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<tr>
<th>Activity assayed</th>
<th>Assay conditions</th>
<th>GS activity [nmol min⁻¹ (mg protein)⁻¹]</th>
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<tr>
<td></td>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Transferase</td>
<td>(a) pH 8, +0.85 mM-alanine</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>(b) pH 8, +0.85 mM-alanine + 60 mM-MgCl₂</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>(c) pH 7, +0.85 mM-alanine</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>(d) pH 8, no alanine</td>
<td>198</td>
</tr>
<tr>
<td>Biosynthetic</td>
<td>Normal assay</td>
<td>10</td>
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<tr>
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<td>−Mg²⁺, 5 mM-Mn²⁺</td>
<td>3</td>
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ND, Not determined.
Fig. 1. Dependence of adenylylated and unadenylylated GSI activity on substrate concentrations. *Rhizobium* strain ANU289 was grown with 10 mM-glutamate as the sole nitrogen source. At mid-exponential phase, the culture was divided in half, and one half was ammonia shocked by the addition of NH$_4$Cl to a final concentration of 15 mM. Both halves were left on the shaker at 28°C for a further 10 min and were then harvested and assayed as described in Methods. Activities are expressed as percentages of the activity of adenylylated GSI at the concentration of substrate normally used in the assay (see Table 1). Each graph is the mean of at least three experiments; the SE was less than 10% of the activity in all cases. O, Adenylylated GSI; ●, unadenylylated GSI.

did result in adenylylation of GSI and that GSI was then deadenylylated by SVPD. The GSI of strain ANU289 appears to differ from other strains in its response to Mg$^{2+}$. The biosynthetic activity of both forms of GSI was also examined. Under normal assay conditions, GSI from ammonia-shocked and untreated cells had the same biosynthetic activity (Table 1). However, when the Mg$^{2+}$ normally present in the assay solution was replaced by 5 mM-MnCl$_2$, a difference was observed, with GSI from ammonia-shocked cells showing the higher activity. This same change in cation specificity, resulting from adenylylation, has also been observed in *Escherichia coli* (Kingdon et al., 1967). It therefore appears that the changes in the properties of GSI resulting from ammonia shock do arise from adenylylation, but there are some differences between GSI from strain ANU289 and other strains. Of particular interest is the observation that the biosynthetic activity of both forms of GSI is the same in ANU289; in the enteric bacteria unadenylylated GSI has high activity while the adenylylated form has virtually none (Kingdon et al., 1967; Bender et al., 1977). This was therefore investigated further by examining the affinities for substrates of the two forms of GSI.

To ensure that we were actually looking at GSI of different adenylylation states in these experiments, the biosynthetic activity in the presence of Mn$^{2+}$ and the transferase activity in the absence of alanine were measured. There were clear differences in these activities between the two forms of GSI (Table 1). Actual $K_m$ values for the three substrates of the biosynthetic reaction could not be determined because GSI showed kinetics characteristic of an allosteric enzyme. However, Fig. 1 shows the dependence of the biosynthetic activity of both forms of GSI on the concentrations of the three substrates – glutamate, hydroxylamine and ATP: no differences between adenylylated and unadenylylated GSI were detectable. GSII had very little biosynthetic activity under any assay conditions.

In strain ANU289, adenylylated GSI had the same biosynthetic activity as unadenylylated GSI in the presence of Mg$^{2+}$ and greater activity in the presence of Mn$^{2+}$. It is therefore misleading to refer to adenylylated and unadenylylated GSI as inactive and active, respectively. Although this seems true for the enteric bacteria, our results show that it is not universal.

*Regulation by ammonia*

The genetic control of various enzymes involved in nitrogen uptake and assimilation was also examined. Initial experiments showed that GSI, GSII, nitrate reductase and an amidase were present at high levels in nitrogen-starved cultures (glutamate as sole nitrogen source). Ammonia was added to these cultures at mid-exponential phase and the activities of these enzymes were
Regulation of GS in Rhizobium

Determined over a period of several days. The effect of the ammonia analogue methylamine (MA) was also determined. Strain ANU289 cannot utilise MA as a nitrogen source for growth, so if MA imitates ammonia in its regulatory effects it could be useful in further studies of nitrogen control. The ammonia concentration chosen was 10 mM, while only 5 mM-MA was used. A lower MA concentration was chosen for two reasons. Firstly, MA is toxic at concentrations above 5 mM and secondly, since MA does not support growth, its metabolism must be blocked at some point. Intermediates of MA breakdown will therefore build up. In Rhizobium strain 32H1, MA is converted to y-N-methylglutamine by GS but no further metabolism occurs (Gober & Kashket, 1983). This may also occur in strain ANU289.

The results of these experiments are shown in Fig. 2. The activities of GSI and amidase were not affected by ammonia, suggesting that they are not under nitrogen control. GSII activity was lost very rapidly, with virtually no activity remaining only 6 h after ammonia addition. Nitrate reductase in strain ANU289 was also repressed by ammonia, although the decline in activity was slower and less complete than that of GSII. MA had the same effect as ammonia on all four enzymes studied. The response of GSII to MA was a little slower than the response to ammonia but this was probably because of the lower MA concentration used. MA also causes adenyllylation of GSI in strain ANU289, in a manner similar to ammonia, further indicating that MA is a good analogue of ammonia.

**Effect of MA on the growth of strain ANU289**

Since MA imitated the regulatory activity of ammonia but did not support growth, it was used to investigate the extent to which nitrogen control occurs in strain ANU289. Strain ANU289 should be unable to grow in the presence of MA on a medium containing a nitrogen source which is broken down by an enzyme under nitrogen control. MA will repress this enzyme, preventing the use of that nitrogen source, but since MA does not support growth the cells will have no alternative nitrogen source and will therefore be unable to grow. The extent to which MA inhibits growth on particular nitrogen sources can therefore be used to determine which enzymes are under nitrogen control.
Table 2. Effect of MA on the growth of Rhizobium strain ANU289 on different nitrogen sources

All experiments were done in liquid N-FGM with the nitrogenous compounds supplied as indicated. 
+++ Normal growth, i.e. no inhibition by MA; +, partial inhibition; 0, total inhibition.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth in the presence of MA (2.5 mM)</th>
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<tr>
<td>Acetamide</td>
<td>+++</td>
</tr>
<tr>
<td>Allantoin</td>
<td>+</td>
</tr>
<tr>
<td>Ammonia</td>
<td>+++</td>
</tr>
<tr>
<td>Aspartate</td>
<td>+</td>
</tr>
<tr>
<td>Glutamate</td>
<td>+</td>
</tr>
<tr>
<td>Glutamine</td>
<td>+</td>
</tr>
<tr>
<td>Histidine</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>+</td>
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<tr>
<td>Urea</td>
<td>+++</td>
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</table>

The effect of 2.5 mM-MA on the growth of strain ANU289 on various nitrogen sources is shown in Table 2. From the results of the previous section, it would be expected that MA would inhibit growth on nitrate but would have no effect on growth on acetamide. This in fact occurred, indicating that MA has no non-specific effects at this concentration and can therefore be used successfully to investigate nitrogen control in this manner. Nitrate was the only nitrogen source where growth was totally inhibited by MA. This suggests that nitrate reductase is completely repressed by MA, in contrast to the results of the previous section where only a 75% loss of nitrate reductase activity occurred in response to ammonia or MA. This may be due to differences in the responsiveness to MA or uptake of MA at different stages of the growth cycle.

Growth on ammonia, acetamide and urea was unaffected by MA while growth on all other nitrogen sources was partially inhibited. Partial inhibition probably indicates that repression of these enzymes by MA was not complete. A higher concentration of MA than 2.5 mM may be required for total repression of these enzymes.

MA could affect either the uptake systems for these nitrogen sources or the enzymes involved in their degradation. The inhibition of growth on glutamine and glutamate appeared to be an uptake effect. Since the sole pathway of ammonia assimilation is through GS and GOGAT, ammonia must be converted to glutamine by GS and then to glutamate by GOGAT. Growth on ammonia was not inhibited by MA, suggesting that MA does not affect the assimilation of glutamine and glutamate. Thus, the uptake systems for glutamine and glutamate may be regulated by the level of ammonia. However, preliminary experiments showed that the uptake of glutamine and glutamate was only slightly repressed by growth on ammonia. We have no explanation for this discrepancy.

DISCUSSION

We have shown that strain ANU289 is capable of efficient ammonia assimilation through the GS/GOGAT pathway, and can grow at high ammonia concentrations. This is in contrast to other reports which have suggested that some Rhizobium strains grow poorly on ammonia (O’Gara & Shanmugam, 1976; Ludwig, 1978; Osburne & Signer, 1980). While this difference may be partly attributable to strain differences it could also be due to the absence of a buffer in the growth media used in previous studies. We have found that several Rhizobium strains grow poorly on ammonia in the absence of a buffer but are capable of rapid growth on ammonia in the presence of 50 mM-MOPS or HEPES. Dilworth & Glenn (1982) have also shown that R. leguminosarum can grow in the presence of up to 90 mM-ammonia in a buffered medium. It has been argued that Rhizobium species have adapted to export ammonia in the bacteroid state and these adaptations result in a reduced ability to assimilate ammonia in the free living state (Ludwig 1978; Osburne & Signer, 1980). This is certainly not true for strain ANU289 and this argument may have to be reconsidered if other strains and species are able to grow on ammonia in a buffered medium.
The regulation of glutamine synthetase in strain ANU289 differed in several major aspects from that in Klebsiella, in a manner which compensated for the lack of GDH. In Klebsiella, high concentrations of ammonia result in both adenylylation and hence inactivation of GSI, and repression of the synthesis of GSI. Thus, all ammonia is assimilated via GDH. In strain ANU289, adenylylation did not significantly inactivate GSI, and GSI was not repressed by ammonia. This suggests that GSI may play a major role in ammonia assimilation in the presence of high ammonia concentrations, thus replacing GDH. Other studies have also found that GSI is not repressed by ammonia (Fuchs & Keister, 1980b; Darrow et al., 1981) but have not looked at the effect of adenylylation.

GSI, however, was strongly repressed by ammonia. The rapid removal of GSI activity in response to ammonia makes it difficult to see how GSI could be important in ammonia assimilation. Similar results have been obtained by Fuchs & Keister (1980b), who found that ammonia caused a rapid loss of GSI activity in several Rhizobium species. They suggested that active removal of GSI must occur under these conditions. GSI from strain ANU289 is also completely repressed in bacteroids (R. Sandeman, personal communication) so it is unlikely to have a role in the symbiotic state. Ammonia repression of GSI also occurs in wild-type R. meliloti (Somerville & Kahn, 1983), but in mutants of R. meliloti lacking GSI, GSI is no longer repressed by ammonia. GSI in Rhizobium may therefore normally assimilate ammonia under some specific and limited conditions which have not yet been determined.

In strain ANU289, MA exerts the same regulatory effects as ammonia both at the level of adenylylation of GSI and at the genetic level. We used MA to show that the utilization of a number of different nitrogen sources was under nitrogen control. However, our results suggest that ammonia caused a rapid loss of GSI activity in several Rhizobium species. They suggested that active removal of GSI must occur under these conditions. GSI from strain ANU289 is also completely repressed in bacteroids (R. Sandeman, personal communication) so it is unlikely to have a role in the symbiotic state. Ammonia repression of GSI also occurs in wild-type R. meliloti (Somerville & Kahn, 1983), but in mutants of R. meliloti lacking GSI, GSI is no longer repressed by ammonia. GSI in Rhizobium may therefore normally assimilate ammonia under some specific and limited conditions which have not yet been determined.

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