# STUDIES ON NITROGENASE EXPRESSION IN RELATION TO GLUTAMINE SYNTHETASE I AND II OF *RHIZOBIUM* MUTANTS

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## ABSTRACT

An inverse relationship between the level of glutamine synthetase (GS) II and adenylylation (Eñ) of GS I was observed in different mutants of *Rhizobium meliloti* 03 The Eñ of GS I was significantly lower in aerobic conditions than in aerobic conditions. The mutants of *R. japonicum* D211 expressed higher En of GS I with lower nitrogenase activity.

## INTRODUCTION

POSITIVE evidence about the role of glutamine synthetase (GS) in the control of nitrogenase synthesis has been well presented[1-4]. It has been found that *R. japonicum* contains two forms of GS, designated[5] as GS I and GS II. GS I is reversibly adenylylated, repressed only two-fold by ammonium ions and is heat stable at 50°C whereas GS II is thermolabile and cannot be adenylylated. GS I plays a major role in nitrogenase regulation, whereas GS II may be required for purine biosynthesis[4 6]. Ludwig[7] reported that nitrogenase in *Rhizobium* sp. 32 H1 is regulated by unadenylylated GS I. Fuchs and Keister[8] identified two distinct GS in *Agrobacterium* and fast-growing *Rhizobium*. It was also found that possession of GS II appeared to be a specific attribute of *Rhizobiaceae*[9]. It has been suggested that in *Klebsiella pneumoniae*, the *hut* and *nif* operons are regulated by the *ntr* genes which are linked to the structural gene for GS[10, 11]. In this paper, nitrogenase expression was studied with respect to the level of GS II and the adenylylation (Eñ) of GS I in different mutants of *R. japonicum* D211.

## MATERIALS AND METHODS

### *Baceterial strains*

*Rhizobium japonicum* D211 was obtained from M. Moreckova, Research Institute of Crop Production, Ruzyne, Prague, Czechoslovakia. *R. meliloti* 03 was isolated from the nodules of *Trigonella foenum-graecum L* in this laboratory.

### *Media*

The stock rhizobial cultures and their mutants were maintained on yeast mannitol medium[12] solidified with 1.5% Davis New Zealand agar. For the asymbiotic nitrogenase assay, the LNB-5 medium was used as described by Kurz and LaRue[13].

### *Asymbiotic nitrogenase assay*

Freshly grown mutants of *R. japonicum* D211 were transferred aseptically onto the LNB-5 slants and incubated for 36 hr at 28 ± 1°C in anaerobic condition. Nitrogen from each tube was replaced by argon gas. One ml of pure acetylene gas was then injected in each tube. After an incubation of 1 hr, 2 ml of gas phase from each tube were drawn and injected into gas chromatograph (CIC Baroda). The reduction of acetylene to ethylene was monitored as described by Kurz and LaRue[13].

### *Glutamine synthetase (EC. 1.6.3.2) assay*

After assaying nitrogenase, the cells were scraped from the LNB-5 slants, washed twice and resuspended in 5 ml of glass distilled water. This was then used for the whole cell assay of glutamine synthetase I and II. Cetyltrimethyl ammonium bromide (CTAB)-treated cells were used for γ-glutamyl transferase assay as described by Bender *et al*[14]. For adenylylation of GS I, 1 ml of distilled water in assay mixture was replaced by 0.8 M $MgCl_2$. For GS II assay, 0.5 ml enzyme aliquots of different samples were treated at 60°C for 15 min (for *R. japonicum*) and at 50°C 15 min (for *R. meliloti* 03) in a water bath. The enzyme system contained 0.8 ml of assay mixture, 0.1 ml of enzyme (whole cells) and 0.1 ml of 0.2 M glutamine, and 2 ml of stopping mixture was added to stop the reaction after an incubation of 30 min at 37°C. γ-glutamyl hydroxamate was measured at 540 nm in a Bausch and Lomb

Spectronic-20 colorimeter. Specific activity of the enzyme was defined as $\gamma$-glutamyl hydroxymate formed per mg protein per minute at 37°C. Adenylylation (Eñ) of GS I was calculated using a formula as described by Ludwig[6],

$$Eñ = 12\left[1 - \frac{\text{activity } (+Mg^{+2})}{\text{activity } (-Mg^{+2})}\right]$$

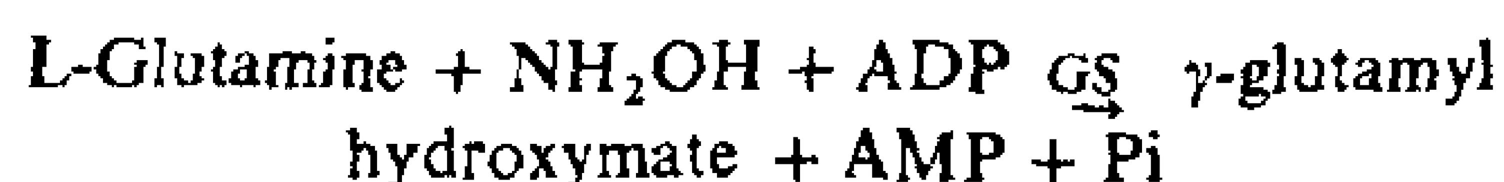
For assaying glutamine synthetases in aerobic conditions, cultures were grown overnight at 28 ± 1°C in liquid LNB-5 medium on a shaker.

*Whole cell protein assay*

One ml sample was boiled with 0.5 ml of 1 N NaOH for 10 min. After cooling, the whole cell protein was assayed by Lowry's method[15].

## EXPERIMENTAL RESULTS AND DISCUSSION

GS I and II were both assayed by $\gamma$-glutamyl hydroxymate 'transferase' reaction as shown in the following equation.



$$\text{L-Glutamine} + NH_2OH + ADP \underset{\rightarrow}{GS} \gamma\text{-glutamyl hydroxymate} + AMP + Pi$$

Shapiro and Stadman[16] have shown that transferase activity in *E. coli* measured in the presence of 60 mM $Mg^{2+}$ was directly proportional to the amount of unadenylylated GS. It varies from unadenylylated (Eñ = 0) to fully adenylylated (Eñ = 12). Therefore, the ratio of transferase activities measured in the presence and absence of $Mg^{2+}$ yields the average adenylylation state (Eñ) for the enzyme[16], at an appropriate pH (7.2) where both forms of the enzyme have equal activity[14]. The same approach was made in the present studies to determine the Eñ of GS I from *Rhizobium* mutants (table 1).

**Table 1** *Relation between GS II and adenylation (Eñ) of GS I in Rhizobium meliloti 03 mutants grown aerobically*

| Strain/Mutants* | % GS II** | Eñ of GS I** |
|---|---|---|
| *R. meliloti* 03 (RM 03) | 36 | 6.6 |
| RM 03, Rif, Amp–1 | 77 | 2.2 |
| RM 03, Rif, Amp–2 | 77 | 3.4 |
| RM 03, Strep, Rif, Amp–1 | 80 | 3.4 |

* Following symbols denote resistance to the antibiotic: Rif = rifampicin, Strep = streptomycin and Amp = ampicillin. These mutants were isolated by ethidium bromide (10 μg/ml for 1 hr) induced mutagenesis.
** GS II activities and Eñ of GS I were calculated as described in materials and methods.

Fuchs and Keister[8] reported that GS II is inactivated by the heat treatment at 50°C for 1 hr. This has been confirmed by George *et al*[17] from this laboratory. Therefore, GS II activities (table 1) were found by substracting specific activities determined in heated samples (*i.e.* GS I) from those obtained in unheated samples (*i.e.* total GS). It was observed that when the % GS II was high, Eñ of GS I was low. This was supported by Ludwig's report[7] about the inverse relationship between the level of GS II and Eñ of GS I. It has been reported from this laboratory that the levels of GS II and the Eñ of GS I were either co-ordinately regulated or that one affected the other[17].

It was interesting to find that anaerobically grown *R. meliloti* 03 mutants also showed an inverse relationship between GS II and Eñ of GS I (table 2). However, the Eñ values were significantly lower as compared to those obtained in aerobic conditions. This can perhaps be attributed to the fact that in anaerobic conditions, $O_2$ is comparatively unavailable, which in turn results in low levels of ATP and low Eñ. It has been found that the GS I deadenylylates prior to the appearance of nitrogenase activity when cultures of slow-growing rhizobia are subjected to microaerobisis[18]. Therefore, further attempts were made to study the correlation if any, between Eñ of GS I and asymbiotic nitrogenase activity using mutants of slow-growing *R. japonicum* D211.

Lower Eñ of GS I was linked with higher nitrogenase activity (table 3). This indicates that adenylylation of

**Table 2** *Relation between GS II and adenylylation (Eñ) of GS I in Rhizobium meliloti 03 mutants grown aerobically and anaerobically*

| | Aerobic growth** | | Anaerobic growth*** | |
|---|---|---|---|---|
| Strain/Mutants* | % GS II | Eñ of GS I | % GS II | Eñ of GS I |
| *R. meliloti* 03 (RM 03) | 36 | 6.6 | 53 | 4.6 |
| RM 03, Kana–2 | 33 | 8.9 | 53 | 0.3 |
| RM 03, Strep, Rif–1 | 25 | 8.3 | 79 | 2.2 |
| RM 03, Strep, Rif–2 | 16 | 10.0 | 75 | 1.0 |

* Following symbols denote resistance to the antibiotic: Kana = Kanamycin, Rif = Rifampicin and Strep = Streptomycin. These mutants were isolated by ethidium bromide (10 μg/ml for 1 hr) induced mutagenesis.
** Grown in the LNB-5 medium on the shaker
*** Grown on slants of the LNB-5 medium under nitrogen gas. Other details are described in the legend to table 1.

**Table 3** *Expression of nitrogenase activity in relation to adenylylation (Eñ) of GS I in Rhizobium japonicum D211 mutants*

| Strain/Mutants* | % GS II | Eñ of GS I | Nitrogenase Activity** nmol $C_2H_2$/ mg protein/hr |
|---|---|---|---|
| *R. japonicum* D211 (RJ D211) | 21 | 1.0 | 17.9 |
| RJ-UV-1 | 11 | 1.0 | 18.1 |
| RJ-EB-46 | 22 | 4.4 | 6.5 |
| RJ-NTG-5 | 31 | 4.7 | 5.5 |
| RJ-AO-30 | 35 | 8.2 | 0 |

* Mutants designation denotes ultraviolet irradiation (UV, 60 s exposure), ethidium bromide (EB, 10 μg/ml for 1 h), NTG (100 μg/ml for 30 min) and acridine orange (AO, 100 μg/ml for 30 min).

** Measured in free-living cultures grown on the LNB-5 medium. Other details are given in the legend to table 1.

GS I determines the degree of nitrogenase expression. Therefore, it seems possible to isolate a *Rhizobium* mutant constitutive in unadenylylated GS I with a constitutive nitrogenase expression.

GS (*gln* A) is known to act as a positive control element in several nitrogen yielding operons[19]. It has also been shown that the products of two more genes *gln* F (*ntr* A)[20] and *gln* G (*ntr* C)[21] are positive transcriptional activators in *nif* operon of *K. pneumoaniae*[10,11].

The results (tables 1–3) suggest that in the systems studied, the role of GS II may not be unequivocal in purine biosynthesis as proposed by Ludwig[7]. If this is assumed to be true then the availability of adenine would determine the Eñ of GS I and this in turn should correspond to the levels of % GS II. However, in no case has this been observed by us (tables 1–3), and hence it is possible that GS II may not be involved in purine biosynthesis but it might have a role as an ammonia assimilatory enzyme. Thus, unlike that of unadenylylated GS I, the role of GS II either in adenylylation of GS I or in ammonia assimilation is yet to be resolved.

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