Characterization of glutamine synthetase from the ammonium-excreting strain HM053 of *Azospirillum brasilense*

Caracterização da glutamina sintetase da estipe excretores de amônio HM053 de *Azospirillum brasilense*

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Abstract

Glutamine synthetase (GS), encoded by glnA, catalyzes the conversion of L-glutamate and ammonium to L-glutamine. This ATP hydrolysis driven process is the main nitrogen assimilation pathway in the nitrogen-fixing bacterium *Azospirillum brasilense*. The *A. brasilense* strain HM053 has poor GS activity and leaks ammonium into the medium under nitrogen fixing conditions. In this work, the glnA genes of the wild type and HM053 strains were cloned into pET28a, sequenced and overexpressed in *E. coli*. The GS enzyme was purified by affinity chromatography and characterized. The GS of HM053 strain carries a P347L substitution, which results in low enzyme activity and rendered the enzyme insensitive to adenylylation by the adenilyltransferase GlnE.

Keywords: nitrogen fixation, adenylylation, adenilyltransferase, EC 6.3.1.2, GlnE.

Resumo

A glutamina sintetase (GS), codificada por *glnA*, catalisa a conversão de L-glutamato e amônio em L-glutamina. Este processo dependente da hidrólise de ATP é a principal via de assimilação de nitrogênio na bactéria fixadora de nitrogênio *Azospirillum brasilense*. A estirpe HM053 de *A. brasilense* possui baixa atividade GS e excreta amônio no meio sob condições de fixação de nitrogênio. Neste trabalho, os genes *glnA* das estirpes do tipo selvagem e HM053 foram clonados em pET28a, sequenciados e superexpressos em *E. coli*. A enzima GS foi purificada por cromatografia de afinidade e caracterizada. A GS da estirpe HM053 possui uma substituição P347L que resulta em baixa atividade enzimática e torna a enzima insensível à adenylylação pela adenililtransferase GlnE.

Palavras-chave: fixação de nitrogênio, adenylation, adenilyltransferase, EC 6.3.1.2, GlnE.

1. Introduction

In plants, nitrogen starvation is associated with reduction of cell division and expansion, leaf area and photosynthesis (Lawlor David, 2002). Plants can use as nitrogen sources ammonium, nitrate and amino acids, but cannot incorporate the most abundant form of nitrogen available on earth, dinitrogen (N₂). Thus, agricultural productivity is heavily dependent of the use of synthetic nitrogen fertilizers, which are expensive and causes severe environment impacts (Ter Steege et al., 2001; Vance, 2001).

Biological fixation of nitrogen is the reduction of dinitrogen gas into ammonium by the nitrogenase complex present in a restricted group of prokaryotes. Amongst other factors, biological nitrogen fixation is negatively controlled by the availability of ammonium (Hartmann et al., 1986; Merrick and Edwards, 1995). Proteobacteria typically assimilate the ammonium through the GS-GOGAT pathway. The glutamine synthetase (GS), encoded by the *glnA* gene, catalyses the conversion of L-glutamate and ammonium to L-glutamine, in a process energetically driven by ATP hydrolysis (Westby et al., 1987). The glutamate synthase enzyme (GOGAT), encoded by the *gltD* and *gltB* genes, catalyses the reductive transfer of the amide group from L-glutamine to α-ketoglutarate, producing two L-glutamate molecules in an NADPH-dependent reaction (Merrick and Edwards, 1995; Westby et al., 1987).

Ammonium assimilation requires cellular energy and is regulated at both the transcriptional and post-translational levels. Transcriptional regulation of *glnA* expression is central to the control of ammonia assimilation (Antonyuk, 2007; Dixon and Kahn, 2004; Leigh and Dodsworth,
2. Materials and Methods

2.1. Bacteria and growth conditions

*E. coli* cells were grown at 37 °C in liquid LB (Sambrook et al., 1989) with shaking at 120 rpm or in LA solid medium (15 g L⁻¹ agar) with appropriate antibiotics.

2.2. Cloning

The glnA gene of *A. brasilense* FP2 (wild type) and HM053 were amplified using the primers shown in Table S1. The PCR products were cloned into the vector pBluescript II KS (+), digested with EcoRV and the inserts completely sequenced. The glnA genes were then transferred to the pET28a vector using the restriction enzymes NdeI and HindIII. The pET28a derivatives were used to express the wild type and mutant (herein named P347L-GS) glutamine synthase synthases of *A. brasilense* in *E. coli* BL21 DE3.

2.3. Purification of glutamine synthetase

*E. coli* BL21 containing the overexpression plasmids were grown overnight in 6 ml of LB medium containing kanamycin at 37 °C and 160 rpm. This pre-inoculum was then poured into 100 ml of LB containing the antibiotic and shaken at 37 °C until an optical density of 0.5, had been reached. Then, to induce protein synthesis 250 μM IPTG was added followed by incubation overnight at 16 °C aerobically. The next day, the cultures were plated on ice for 30 min, then collected by centrifugation (15 min, 5000 g, 4 °C) and re-suspended in 20 ml of lysis buffer (150 mM NaCl and 50 mM Tris-HCl, pH 8). Cells were lysed by sonication in an ice bath (15 cycles, 15 sec on/15 sec off).

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Bio-Sciences, Pittsburgh, PA 15264-3065, USA) coupled to a peristaltic pumping system. The proteins were eluted in buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl) with increasing gradient of imidazole from 10 mM to 1 M. Purified proteins were quantified by the Bradford method (Bradford, 1976) and the purity was checked on SDS-PAGE gels using the ImageJ program.

2.4. Identification of glutamine synthetase by MALDI-TOF mass spectroscopy

Samples were prepared for analysis according to Shevchenko et al. (1996) and analyzed in a MALDI-TOF Autoflex II spectrometer (Bruker Daltonik GmbH, Life Sciences, 28359 Bremen, Germany). Lists of peaks were created using FlexAnalysis 3.0 software (Bruker Daltonik). Protein identification was performed using the Mascot 2.2 software and the protein database of A. brasilense sp245 (Wisniewski-dyé et al., 2011).

2.5. Electrophoresis and western blot assays

Electrophoresis and western blot assays were performed according to Huergo et al. (2006), with an anti-GS antibody diluted 10,000 fold (van Heeswijk et al., 1996).

2.6. Transferase activity of glutamine synthetase

Transferase activity was assayed according to Bender et al. (1977), with some modifications. HEPES 100 mM was used instead of imidazole hydrochloride and the total reaction volume was reduced to 302.5 µl (10 µL sample, 80 µL mix, 12.5 µL l-glutamine and 200 µL stop mix).

To determine the physiologically active (non-adenylated) fraction of GS, the system was supplemented with 60 mM MgCl₂ which inhibits the adenylylated fraction (physiologically inactive). A pH (7.66) was assumed for the iso-electric point of both the adenylylated and unadenylated forms (Machado et al., 1991).

2.7. Phosphodiesterase treatment

The GS used in Western Blot assays was treated with commercial snake venom phosphodiesterase (Merck) according to Pirola et al. (1992), with some modifications. The reactions containing GS (0.3 µg) 0.03 µg of phosphodiesterase in 10 µL in 10 mM Tris-HCl pH 8 and 5 mM MgCl₂ were incubated at 30 °C for 1 h. The snake venom phosphodiesterase (SVP) was dissolved in 20 mM Tris-HCl (pH 8) at a concentration of 1 µg.µl⁻¹.

2.8. Modelling the Structure of A. brasilense glutamine synthetase

Structural prediction was performed using the Swiss-model server (Waterhouse et al., 2018). The Pymol program (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used to compare wild-type GS with the mutant P347L-GS.

3. Results

3.1. Purification of glutamine synthetase

GS was purified from E. coli BL21 by affinity chromatography. SDS-PAGE electrophoresis of the purified fraction showed a band of ~ 60 kDa (Supplementary Material - Figure S1 and Table S2). The wild-type GS was more than 90% homogeneous while the P347L-GS was about 80% homogeneous. The concentrations of wild-type GS obtained were typically 1 µg.µl⁻¹ while those of P347L-GS were considerably less (0.1 µg.µl⁻¹).

3.2. Identification of GS by mass-spectroscopy (MALDI-TOF)

MALDI-TOF mass-spectroscopy confirmed that the purified proteins were GS of A. brasilense (see Table 1). Further proof that the purified proteins was indeed GS, came from western blot analyses (data not shown) which also allowed the separation of the unmodified and adenylylated subunits. An AMP group changes the rate of migration in electrophoreses gels, causing the band to migrate more slowly than non-adenylated form (Bender and Streicher, 1979), for this reason, a double-band pattern of GS was visible on the SDS-PAGE gels.

3.3. Assay of transferase activity

GS transfers the glutamyl radical from L-glutamine to hydroxylamine producing γ-glutamyl-hydroxamate. Depending on the assay conditions used either the non-adenylated form is active and catalyzes the transfer (in the presence of low Mn²⁺ and high Mg²⁺) or both non-adenylated and adenylylated forms are active (presence of low Mn²⁺ and absence of Mg²⁺) (Bender et al., 1977). The assay used here determines the fraction of physiologically active (non-adenylated) GS. Since the two GS forms have different optimum pHs, the reaction was carried out at the iso-active point (pH 7.66) (Machado et al., 1991).

The wild-type GS had high total activity (+ Mg²⁺) and low non-adenylated activity, suggesting that the purified enzyme was heavily adenylylated (see Figure 1A and B). This was expected since the growth medium used to cultivate the overproducing strain rich in nitrogen source. In contrast, P347L-GS had very little activity under both conditions (1,000 times lower than wild-type GS for total activity), suggesting that the P347L mutation drastically affects enzyme activity.

Snake-venom phosphodiesterase (SVP) catalyses the removal of the AMP moiety from GS. When added to

| Sample          | Protein identified | Mascot MOWSE Score | M.W. (kDa)* | # Peptides identified | Coverage |
|-----------------|--------------------|--------------------|-------------|----------------------|----------|
| Wild-type GS    | Glutamine synthetase | 131                | 52.3        | 12                   | 38%      |
| P347L-GS        | Glutamine synthetase | 141                | 52.3        | 13                   | 33%      |

*Molecular weight.
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Purified GS (in the presence of Mg\(^{2+}\)), transferase activity was restored in full, confirming that the purified GS was heavily adenylylated (see Figure 1A). Again, P347L-GS behaved differently - treatment with phosphodiesterase did not alter its activity in under either condition (see Figure 1B).

3.4. Western blot analyses

Western blot analyses were also performed before and after digestion with SVP. Wild type GS responded to the treatment (see Figure 2A). Initially the protein was fully adenylylated (one slowly migrating band) but with time the protein lost adenylyl residues as judged by the concomitant appearance of faster migrating band. At the same time, its enzyme activity increased (Figure 1A). In contrast, treatment of P347L-GS with SVP (see Figure 2B) did not affect the protein migration rate nor its activity (see Figure 1B).

3.5. Structural prediction

Structural models for *A. brasilense* GS and the P347L variant were generated using the Swiss-model server (Waterhouse et al., 2018) with crystal structure of the *Salmonella typhimurium* GS (Gill and Eisenberg, 2001) and analyzed using the Pymol program (see Figure 3A). Estimation of the quality of the predicted model using the Global Model Quality Estimation (GMQE) was 0.82 for the wild-type GS and 0.81 for P347L-GS. The Root Mean Square Deviation (RMSD) for the alignment of the structural models was 1.296 Angstroms (Å), with all-atom (no outlier rejection) and without superposition. This comparison revealed that the variant P347L most probably affects the secondary structure of the protein at amino acids 352 (P), 353 (K), and 354 (G). In P347L-GS, this region forms an alpha-helix that is absent from the wild type GS (see Figure 3B). Amino acid 354 interacts in a polar fashion with 351 (S), and 356 (R) in both forms of GS. Another difference possibly caused by the predicted structure of the three amino acids (352-354) is in the position of arginine 356. Overlap of the two structure models showed a difference in the position of this residue of 0.8 Å.

![Figure 1](image1.png)

**Figure 1.** Transferase activity of glutamine synthetase. (A) Transferase activity of wild-type glutamine synthetase in the absence and presence of magnesium as well as with snake venom phosphodiesterase treatment; (B) Transferase activity of P347L glutamine synthetase in the absence and presence of magnesium, and with snake venom phosphodiesterase treatment. The activity of GS is expressed in µmol γ-glutamyl-hydroxamate min\(^{-1}\) mg protein\(^{-1}\), given that the absorbance of 530 nm of 1 µmol γ-glutamyl-hydroxamate was 0.054. The total activity was determined in the absence of Mg\(^{2+}\) (-Mg\(^{2+}\)) and the non-adenylylated (active) fraction was determined in the presence of 60 mM Mg\(^{2+}\) (+Mg\(^{2+}\)). Samples were incubated at 30 ºC for 0, 10, 30 and 60 min before measuring activity. SVP-treated GS samples (+ SVP) were incubated with snake venom phosphodiesterase. GS activity reactions contained 3 µg of protein.

![Figure 2](image2.png)

**Figure 2.** Western blot assays of glutamine synthetase after treatment with snake venom phosphodiesterase. Samples (~ 0.3 µg GS protein) were separated by SDS-PAGE followed by Western blotting with an anti-GS antibody. A) Wild-type glutamine synthetase; B) P347L glutamine synthetase. Lane 1: GS after 0 min of incubation at 30 ºC without any treatment; lanes 2 to 5: GS after 0, 10, 30 and 60 min incubation at 30 ºC with snake venom phosphodiesterase. Lane 6: GS after 60 min incubation at 30 ºC without treatment.
4. Discussion

Due to its higher solubility, much larger amounts of purified wild type GS were obtained than was the case for P347L-GS. Visual comparisons of SDS-PAGE gels of protein extracts made from cultures induced for 18 h at 16°C, suggest that wild-type GS was ~50% soluble, whereas the mutant was almost completely insoluble. Since the purification method used was for soluble proteins, this would explain the difference in the quantities and qualities of proteins obtained.

Our results are in perfect agreement with previous findings. Machado et al. (1991) worked with the A. brasilense strain HM053 and tested GS activity in vivo under three conditions with cells cultured in minimal medium containing 5 mM glutamate, 2 mM NH₄⁺ or 20 mM NH₃⁻ as nitrogen source. Under these conditions, the wild-type strain showed GS activity as between six and fifteen times more active than the GS detected in the HM053 strain. Similar experiments were also performed by Vitorino et al. (2001), who confirmed the higher activity of wild-type GS.

Machado et al. (1991) neither used purified proteins nor knew exactly how much enzyme was present in the assays. Our work with purified GS proved that the specific activity of the P347L-GS present in the HM053 strain was in fact much lower. The low GS activity is likely to restrict NH₄⁺ assimilation and glutamine production in the HM053 strain thereby resulting in Nif⁺ phenotype. The low GS activity would result in low intracellular L-glutamine levels and in the phosphoryl transfer from ATP to glutamate, and of the negatively charged substrates, glutamate and ATP, the active and, along with n2, participates in the binding of the negatively charged substrates, glutamate and ATP, and in the phosphoryl transfer from ATP to glutamate, and then the glutamyl transfer from glutamate to ammonia (Ginsburg and Stadtman, 1973; Liaw et al., 1993a, b; Liaw et al., 1994b).

The change P347L also seems to be affecting the structural stability of GS as judged by the reduced solubility release by diffusion to the cell membrane to the culture medium (Santos et al., 2017).

Snake-venom phosphodiesterase was able to de-adenylate the wild type GS (Johansson and Gest, 1977). The P347L-GS variant showed a different behaviour upon treatment with SVP. P347L-GS does not appear to be adenylated in vivo. Western blotting confirmed that purified P347L-GS was not adenylated. Somehow, the P347L change of HM053 prevents GS adenyllylation by the ATase while reducing the activity of the non-adenylated form (Machado et al., 1991; Santos et al., 2017). Modelling of the P347L-GS structure and comparison with the wild type A. brasilense GS did not reveal remarkable structural differences, except for the secondary structure of the region between the residues 352-354 and a 0.8 Å shift in the position of arginine 356. This result suggests that the point mutation is not affecting directly the active sites of GS. On the other hand, the arginine residue at position 356 does a polar interaction with histidine at position 272 (homologous to H-271 in S. typhimurium (Castellen et al., 2009), which coordinates the α-phosphate group of ADP/AMPPNP and E-129 (Liaw et al., 1994b). The shift in R-356 position in GS-P347L changes slightly the bond length and bond angle with H-272, which may affect indirectly the active site. Glutamate at position 129 (homologous to E-131 in A. brasilense (Castellen et al., 2009) does hydrogen bonds with H-271, and coordinates the n2 ion (Liaw and Eisenberg, 1994a). GS contains two divalent cation sites (n1 and n2) and one monovalent cation site at the subunit interface. Ions that occupy these three ion sites are needed for GS activity. The n1 metal ion stabilizes the enzyme in the active and, along with n2, participates in the binding of the negatively charged substrates, glutamate and ATP, and in the phosphoryl transfer from ATP to glutamate, and then the glutamyl transfer from glutamate to ammonia (Ginsburg and Stadtman, 1973; Liaw et al., 1993a, b; Liaw et al., 1994b).
of the mutant form and absence of adenylylation. However, we could not observe in our model changes that could account for these effects.

In summary we demonstrated that the properties of the mutant form of GS in strain HM053 can explain the constitutive expression of nitrogenase and ammonium excretion, and that moduletion of GS activity in nitrogen-fixing bacteria can decouple the tight control of nitrogenase expression and activity allowing excretion of ammonium.

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Supplementary Material

Supplementary material accompanies this paper.
Figure S1. 10% SDS-PAGE gel of purified glutamine synthetase.
Table S1. Table of primers used to amplified the glnA genes of A. brasilense FP2 (wild type) and HM053.
Table S2. Table of purification of glutamine synthetase.
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