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Azospirillum brasilense glutamine synthetase: influence of the activating metal ions on the enzyme properties

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The kinetic properties of the Mg^{2+}-activated and Mn^{2+}-activated glutamine synthetase (GS) of *Azospirillum brasilense* in the biosynthetic reaction were studied. The Mg^{2+}-supported and Mn^{2+}-supported GSs in an average state of adenylylation varied in pH optimum, maximum activity, saturation functions for ammonium and glutamate, affinity to substrates, and in the Me^{2+}-ATP ratio required for the optimal enzyme activity. Seventeen other cations were tested for the maintenance of GS activity. The level of the latter and the kinetic behavior of the GS in *A.brasilense* is suggested to depend essentially on the concentrations of Mg^{2+}, Mn^{2+} and Co^{2+}, as well as on their ratio.

Keywords: *Azospirillum brasilense*, Glutamine synthetase, Metals

Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase

Introduction

Glutamine synthetase (L-glutamate: ammonia ligase (ADP-forming), EC 6.3.1.2.; GS), which catalyzes the ATP-dependent synthesis of glutamine from glutamate and ammonium, is a key enzyme of nitrogen metabolism in the bacterial cell. The vital importance of the bacterial GS is dictated by the following: *(i)* the GS reaction is the only pathway for glutamine synthesis; *(ii)* the amide group of glutamine is used in the syntheses of purines and pyrimidines, some amino acids, glucosamine-6-phosphate and *p*-aminobenzoic acid (Meister 1980); *(iii)* the GS-GOGAT pathway is the primary way for ammonia assimilation in *Azospirillum brasilense* and other bacteria (Westby *et al.* 1987); *(iv)* GS and its structural gene *glnA* are closely related to the regulatory system that controls nitrogen metabolism (Magasanik & Neidhardt 1987; Zhang *et al.* 1997). Obviously, regulation of an enzyme with such intricate functions has to be complex.

GS has been isolated from a number of microorganisms (Meister 1980; Dang Khoang Fyok Khien *et al.* 1988; Pushkin 1990; Pirola *et al.* 1992), the most extensively studied being the enzyme from *Escherichia coli*. The *E. coli* GS consists of 12 identical subunits of 50 kDa each, arranged in 2 hexagonal rings (Stadtman *et al.* 1980). This enzyme and some other bacterial GSs are regulated by means of covalent modification (adenylylation and deadenylylation) of each subunit. This process is performed by the enzyme adenylyltransferase, the activity of which is modified by uridylylation-deuridylylation of the regulatory protein P_{II}. The bicascade system of activity regulation enables the kinetic properties of GS to be finely regulated. Thus, the extent of

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adenylylation affects the enzyme activity, its specificity to metals, sensitivity to feedback inhibitors, so that, depending on the number of adenylylated subunits and their relative position, the number of multiple molecular forms of the enzyme can reach 382 (Stadtman *et al.* 1980). For bacterial GSs, some other types of regulation are also known (Pushkin 1990). The mechanism of regulation of the *E. coli* GS has been recognized as one of the most complicated among several thousands of enzymes known to date (Lehninger 1993).

As for the GS of *A. brasilense*, a bacterium known to colonize roots of higher plants and to stimulate plant growth, only a few papers dealing with this enzyme have so far appeared. Thus, the primary structure of the enzyme has been deduced from the nucleotide sequence of the *glnA* gene (Bozouklian & Elmerich 1986). The GS from *A. brasilense* has been purified and partially characterized (Pirola *et al.* 1992; Bespalova *et al.* 1994). The molecular weights of the holoenzyme, the adenylylated and deadenylylated subunits were determined to be 630 kDa, 53 kDa and 52 kDa, respectively (Pirola *et al.* 1992). From this it follows that in its molecular organization the *A. brasilense* GS has analogy to the enzyme from enteric bacteria. Recently, it was shown that the transcriptional regulation of the enzyme has some unusual features and differs from the *E.coli* system (de Zamaroczy & Elmerich 1998). The first data for the catalytic properties of the *A. brasilense* GS (Pirola *et al.* 1992; Bespalova *et al.* 1994) revealed complexity of the kinetic behavior and its dependence on at least two factors, *viz*., the extent of adenylylation and the activating cation (Bespalova *et al.* 1994). Also, we have recently studied assimilation of a number of metal ions by *A. brasilense* (Kamnev *et al.* 1997a), as well as their influence on the spectroscopic characteristics of the bacterial cells as a whole (Kamnev *et al.* 1997b) and bacterial membranes (Kamnev *et al.* 1997c).

The goals of this study were to compare the kinetic behavior of the Mg^{2+}-activated and Mn^{2+}-activated GSs and to elucidate which other metal cations are able to support the activity of the *A.brasilense* GS.

Materials and methods

A. brasilense SP245 culture (Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences) was grown in an ANKUM-2M fermenter with stirring for 18 h at 32 °C using the following synthetic medium (g/l): K_2HPO_4, 3.0; KH_2PO_4, 2.0; NH_4Cl, 5.0; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.002; malic acid, 5.0; yeast extract, 0.1; initial pH 6.86.

The GS of *A. brasilense* SP245 was purified as described previously (Bespalova *et al.* 1994) and was electrophoretically homogeneous. The GS activity was measured in the biosynthetic (Deuel & Stadtman 1970) and transferase reactions. For the biosynthetic assay, the reaction mixture (final volume 1.8 ml) contained 100 mM Tris-HCl or MES-NaOH buffer. The concentrations of the other reagents are given in the legends. The reaction was initiated by adding 0.65 mg of the enzyme and allowed to proceed for 15 min at 37°C. The enzyme activity was assayed by estimation of the amount of inorganic phosphate which was determined using a calibration curve. The transferase assay mixture (final volume 0.5 ml) contained 100 mM Tris-HCl buffer, 70 mM L-glutamine, 0.2 mM ATP (sodium salt), 20 mM sodium arsenate, 0.4 mM $MgCl_2$ and 5mM NH_2OH. The reaction was initiated by adding 0.1 ml of the enzyme preparation. After incubation for 15 min at 37°C, γ-glutamyl hydroxamate was determined from a calibration curve. The extent of GS adenylylation was determined by the method of Shapiro and Stadtman (1970). The transferase activity of the enzyme was assayed in the presence and absence of 75 mM $MgCl_2$ as described above, and the extent of GS adenylylation was calculated with the formula proposed by the authors of the method (Shapiro & Stadtman, 1970).

Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

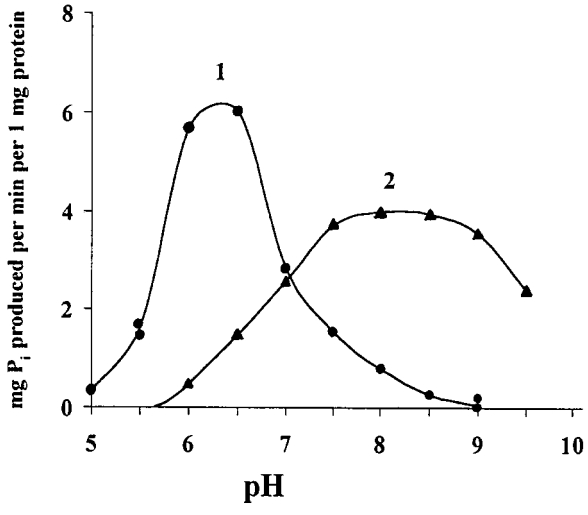

Figure 1. Influence of pH on the activity of the *A. brasilense* SP245 GS in the presence of Mn^{2+} (1) and Mg^{2+} (2).The assay mixture contained 3 mM ATP, 6 mM $MgCl_2$ or $MnCl_2$, 20 mM sodium L-glutamate and 10 mM NH_4Cl.

Results and discussion

It is common knowledge that glutamine biosynthesis involves divalent metal cations, Mg^{2+} and Mn^{2+} being the most effective (Meister, 1980). Such an importance of the ions is caused primarily by the fact that ATP may manifest its substrate property only in a complex with a cation. For GSs regulated by adenylylation-deadenylylation, their affinity to cations is strongly dependent on the enzyme adenylylation extent (Ginsburg *et al.* 1970; Pushkin 1990). The enzyme preparation under study was in an average state of adenylylation (n = 6.4) and exerted its activity with both cations (Fig.1). As can be seen from the figure, the Mg^{2+}-supported GS activity was pronounced in alkaline and neutral regions (with the optimum at pH 8.0), while the Mn^{2+}-supported activity was higher in acidic and neutral regions (with the optimum at pH 6.25). It is interesting to note that at pH 7.1, which has recently been reported as the intracellular pH of *A. brasilense* (Zhulin *et al.* 1996), the Mg^{2+}-supported and Mn^{2+}-supported activities of the enzyme were very close.

We investigated the substrate saturation functions of the Mg^{2+}-activated and Mn^{2+}-activated GS and found them significantly different. With both cations, the saturation functions for ammonia were complex and had a non-Michaelis-Menten nature (Fig. 2a,c). In the case of Mg^{2+}, the maximum GS activity was achieved at a relatively low ammonium concentration, and its further increase resulted in the inhibition of the enzyme.

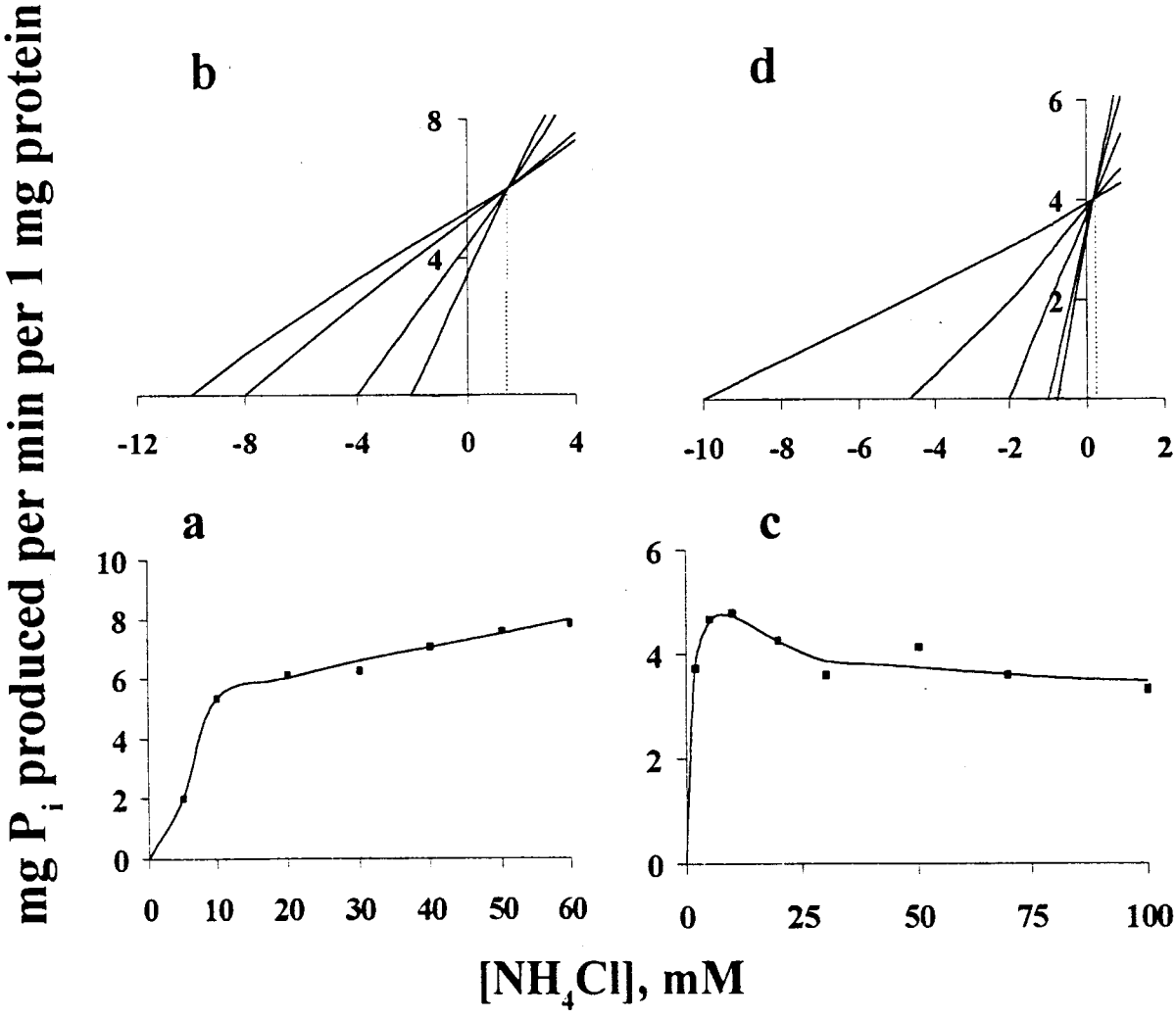

Figure 2. Substrate saturation curves for ammonium studied with Mn^{2+} (a) and Mg^{2+} (c). The same data presented in the Cornish-Bowden coordinates are above the respective substrate saturation curves (b, d). The assay mixture contained 3 mM ATP, 6 mM $MgCl_2$ or $MnCl_2$ and 20 mM sodium L-glutamate; pH 8.0.

The double reciprocal plot was concave (not shown) and precluded the determination of the apparent K_m. A graphic representation of the part of the saturation curve, where substrate inhibition was not observed, using the Cornish-Bowden (1976) method revealed the apparent K_m for ammonium being 0.2 mM, reflecting a rather high affinity of the *A. brasilense* GS for ammonium (Fig. 2d). Replacing Mg^{2+} with Mn^{2+} brought about an abrupt change in the catalytic properties of the enzyme. Thus, saturation for ammonium was not observed even at large non-physiological concentrations of the substrate (Fig. 2a). The apparent K_m underwent a rise from 0.2 to 1.5 mM, that is, the affinity of GS for ammonium decreased 7.5-fold (Fig. 2b).

The Mg^{2+}-activated GS of *A. brasilense* also exhibited substrate inhibition by another substrate, L-glutamate (Fig. 3c). Glutamate caused a greater substrate inhibition than ammonium (35% against 28%). The apparent K_m of Mg^{2+}-activated GS for glutamate determined by the Cornish-Bowden graphic method was 2.3 mM (Fig. 3d). Conversely, when Mn^{2+} was used to activate the enzyme, saturation for glutamate was not reached as in the case of ammonium (Fig. 3a). Neither the double reciprocal plot (not shown) nor the data from the Cornish-Bowden coordinates (Fig. 3b) enable one to determine the apparent K_m value. The latter method yielded a few intersection points, each of which giving a value that was over 2.3 mM glutamate. On this basis, we suggest that the Mg^{2+}-activated *A. brasilense* GS has a higher affinity for glutamate than the Mn^{2+}-activated GS.

The activity of GSs from various organisms is known to depend upon how much the concentration

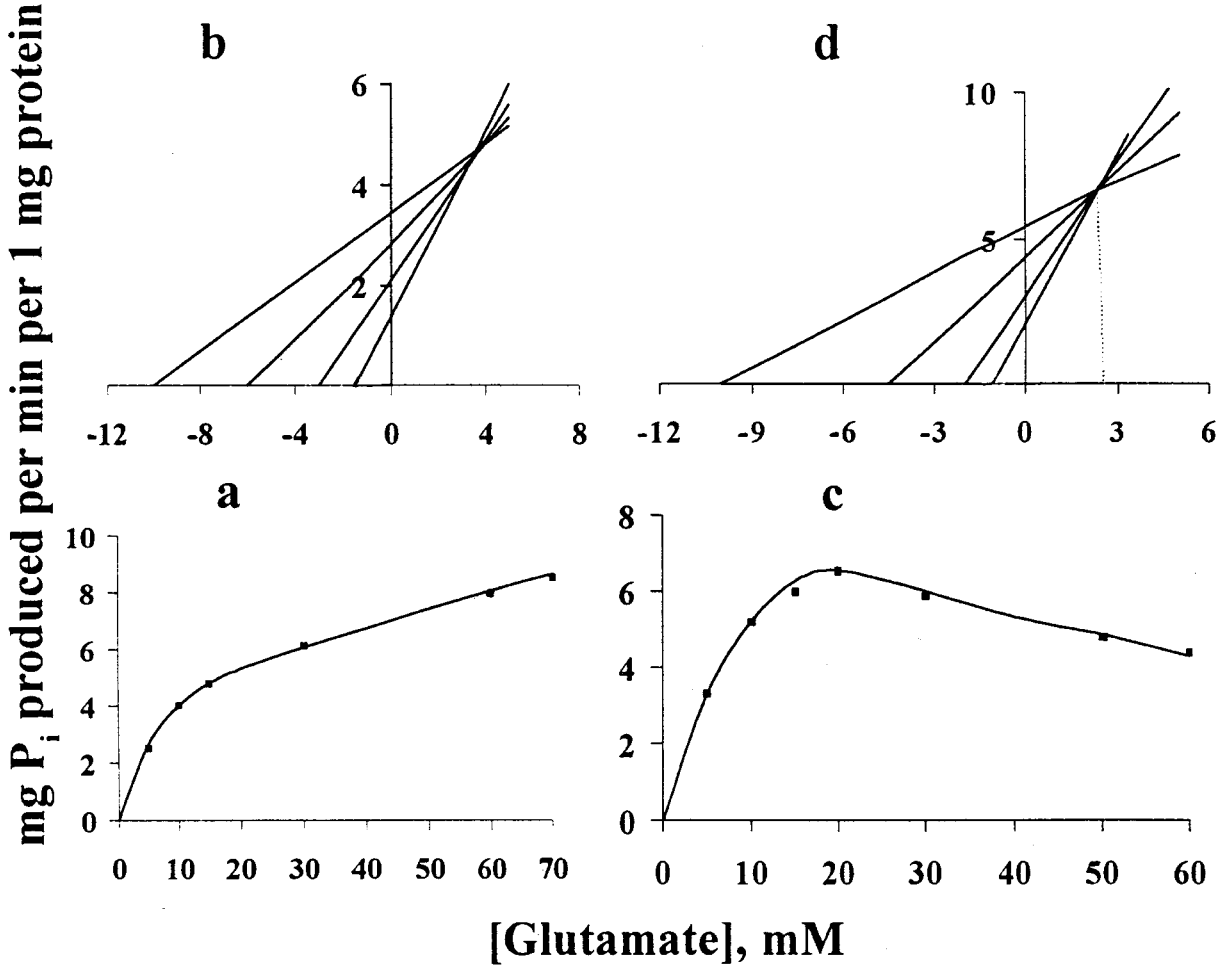

Figure 3. Substrate saturation curves for L-glutamate studied with Mn^{2+} (a) and Mg^{2+} (c). The same data presented in the Cornish-Bowden coordinates are above the respective substrate saturation curves (b, d). The assay mixture contained 3 mM ATP, 6 mM $MgCl_2$ or $MnCl_2$ and 20 mM NH_4Cl; pH 8.0.

of Mg^{2+} or Mn^{2+} is in excess of the ATP concentration (Deuel & Stadtman 1970, Hatanaka *et al.*1987, Pushkin 1990). This point is important, as it is considered that the magnesium and manganese cations, apart from being involved in the formation of the Me^{2+}-ATP complexes, play a role in maintaining the enzyme conformation and also in folding and assembly of the bacterial GS (Pushin 1990, Fisher 1998). In the case of the *A. brasilense* GS, the maximum of the Mn^{2+}-supported GS activity was observed when the ratio of Mn^{2+} to ATP was 2.0 and decreased with any deviation from this ratio (Fig. 4a). By contrast, a significant excess of the Mg^{2+} concentration over the ATP concentration is required for the maximum activity of the *A. brasilense* GS (Fig. 4b). It is notable that in this respect the *A. brasilense* GS in an average state of adenylation is similar to the *Bacillus subtilis* GS which is not regulated by adenylylation-deadenylylation (Deuel & Stadtman 1970).

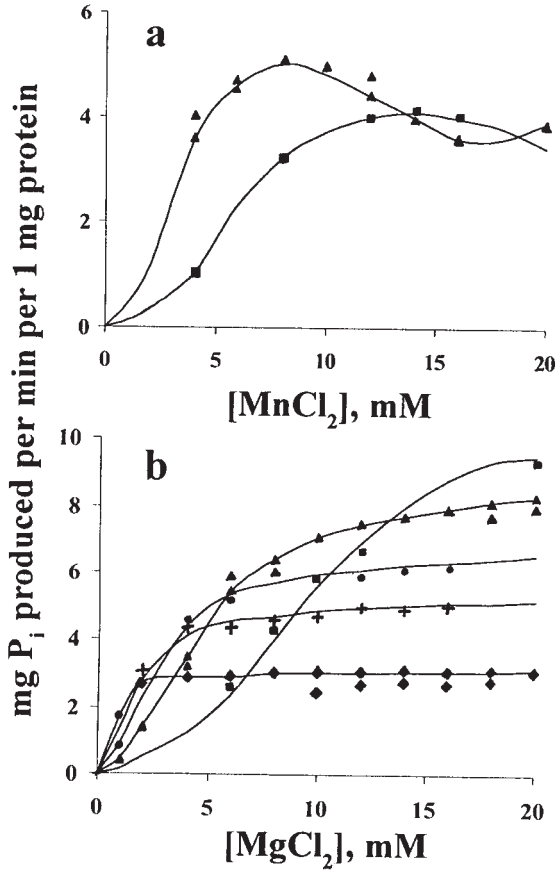

Figure 4. Effect of the Mn^{2+} (a) and Mg^{2+} (b) concentrations on the *A.brasilense* SP245 GS reaction rate at different ATP levels. Concentrations of ATP were 4 mM (▲) and 8 mM (■) for Mn^{2+} and 0.5 mM (◆), 1 mM (+), 2 mM (●), 4 mM (▲) and 8 mM (■) for Mg^{2+}. The assay mixture contained 20 mM sodium L-glutamate and 10 mM NH_4Cl; pH 8.0.

Seventeen other cations at a concentration of 1 mM were tested for the maintenance of the activity of the *A. brasilense* GS (Fig. 5). The data obtained were compared with the activity of GS supported with 1 mM Mg^{2+}. Most of the cations were substantially less efficient in the maintenance of the GS activity than Mg^{2+} (38% to 5% of that for Mg^{2+}). Cu^{2+} and Sn^{2+} did not at all maintain the activity of the enzyme. With Co^{2+}, the GS activity was slightly below that with Mg^{2+}. Moreover, the addition of 1 mM Co^{2+} increased the Mg^{2+}-supported activity of the *A. brasilense* GS more than 3-fold (Fig. 5). The marked enhancement of the activity of bacterial GSs under Mg^{2+}-unsaturated conditions was reported (Segal & Stadtman 1972; Hatanaka *et al.* 1987; Dang Khoang Fyok Khien *et al.* 1988). Thus, in the case of bifidobacterial GSs, the addition of 3 mM Co^{2+} to the biosynthetic reaction mixture containing 4 mM Mg^{2+} resulted in a 3- to 7-fold increase in the enzyme activity, and this stimulation was not due to the increase in ionic strength (Hatanaka *et al.* 1987).

In general, the kinetic behavior of the *A. brasilense* GS is similar to that of other bacterial GSs. Thus, it reacted optimally at pH 6.25 (Mn^{2+}-activated enzyme) and at pH 8.0 (Mg^{2+}-activated enzyme), and these values were in the pH optimum range (6.0–8.5) reported for GSs from other bacteria (Deuel & Stadtman 1970; Hatanaka *et al.* 1987). Saturation functions for the substrates are complex and have a

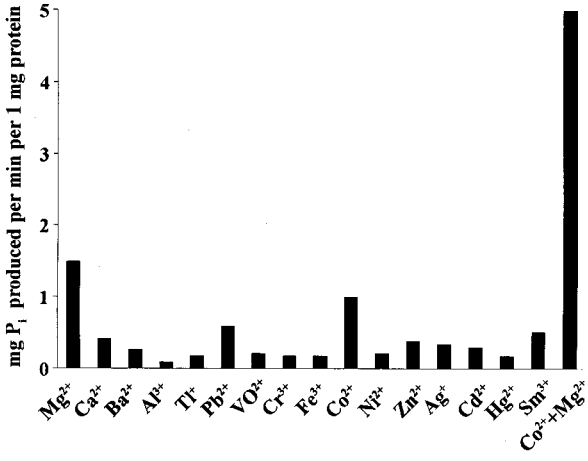

Figure 5. Effect of cations on the *A. brasilense* SP245 GS activity. The assay mixture contained 4 mM ATP, 1 mM $MgCl_2$ or another salt, 10 mM NH_4Cl and 20 mM sodium L-glutamate; pH 8.0.

non-Michaelis-Menten nature both in the case of the *A. brasilense* GS and in the case of GSs from other organisms (Deuel & Stadtman 1970; Hatanaka *et al.* 1987, Pushkin 1990). Among the special features of the azospirillum GS in an average state of adenylylation are lower K_m values for ammonia and glutamate (Deuel & Stadtman 1970; Bhandary & Nicholas 1984; Hatanaka *et al.* 1987). Another distinctive property of the GS under study is a decline in the affinity of the enzyme to ammonium and glutamate when Mg^{2+} in the biosynthetic reaction mixture is replaced with Mn^{2+}.

Of special interest is the specificity of the *A. brasilense* GS towards cations. It is generally recognized that GS is an enzyme with a high specificity for divalent cations (Meister 1980). Thus, the enzyme from *Spirulina platensis* was reported to be activated only with Mg^{2+} and Co^{2+} whereas eight other cations including Mn^{2+} did not support the activity of the *Spirulina platensis* GS (Dang Khoang Fyok Khien *et al.* 1988). The enzyme from *A. brasilense* appeared to show rather a broad specificity towards cations (Fig. 5).

It should be remarked that all the examined kinetic properties of the *A. brasilense* GS *(viz.,* pH optimum, maximum activity, saturation functions for ammonium and glutamate, affinity to substrates, and the Me^{2+}-ATP ratio required for the optimal enzyme activity) were different with Mg^{2+} and with Mn^{2+}. These findings, put together, make it possible to suppose that unadenylylated subunits of the *A. brasilense* GS have an absolute requirement for one cation, whereas adenylylated subunits are specifically activated by the other cation, as it is with the *E.coli* GS, adenylylated subunits of which are activated by Mn^{2+} and unadenylylated ones require Mg^{2+} (Ginsburg *et al.* 1970). This assumption allows an understanding of the pronounced distinctions in the kinetic behavior of the Mg^{2+}-activated and Mn^{2+}-activated azospirillum GSs: when the biosynthetic reaction mixture contains Mg^{2+} as the only metal, unadenylylated subunits only are activated, and when the mixture contains manganese ions, only adenylylated subunits are active and provide for the enzyme activity. It is self-evident that the above assumption requires further experimental verification.

The presented evidence invokes in favor of an important role of divalent cations in the regulation of the *A. brasilense* GS in an average state of adenylylation. It seems reasonable to propose that the activity and the kinetic behavior of the enzyme depend essentially on the concentrations of Mg^{2+}, Mn^{2+}, and Co^{2+}, as well as on their ratio.

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References

Bespalova LA, Korshunova, VE, Antonyuk LP, Ignatov VV. 1994 Isolation, purification and some kinetic properties of moderately adenylylated glutamine synthetase from *Azospirillum brasilense* Sp 245. *Biochemistry* (Moscow) **59**, 41–45

Bhandary B, Nicholas DJD. 1984 Some kinetic properties of a purified glutamine synthetase from bacteroids of *Glycine max. FEMS Microb Lett* **25**, 129–131

Bozouklian H, Elmerich C. 1986 Nucleotide sequence of the *Azospirillum brasilense* Sp7 glutamine synthetase structural gene. *Biochimie* **68**, 1181–1187

Bradford M. 1976 A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254

Cornish-Bowden A. 1976 Principles of Enzyme Kinetics, London: Butterworths.

Dang Khoang Fyok Khien, Solovieva NA, Evstigneeva ZG, Kretovich WL. 1988 Specificity and regulation by metals of *Spirulina platensis* glutamine synthetase. *Doklady Biochemistry* (Moscow) **302**, 984–987.

de Zamaroczy M, Elmerich C. 1998 Regulatory role of the structural gomologues P_{II} and P_Z proteins in *Azospirillum brasilense.* In: Elmerich C, Kondorosi A, Newton WE, eds. Biological Nitrogen Fixation for the 21st Century. Current Plant Science and Biotechnology in Agriculture, vol. 31. Dordrecht: Kluwer Academic Publishers: 111–114.

Deuel TF, Stadtman ER. 1970 Some kinetic properties of *Bacillus subtilis* glutamine synthetase. *J Biol Chem* **245**, 5206–5213

Fisher MT. 1998 GroE chaperonin-assisted folding and assembly of dodecameric glutamine synthetase. *Biochemistry* (Moscow) **63**, 453–472

Ginsburg A, Yen J, Hennig SB, Denton MD. 1970 Some effects of adenylylation on the biosynthetic properties of the glutamine synthetase from *Escherichia coli. Biochemistry* **9**, 633–649

Hatanaka M, Tachiki T, Furukawa R, Toshikura T. 1987 Purification and some properties of glutamine synthetases from bifidobacteria. *Agric Biol Chem* **51**, 425–433

Kamnev AA, Renou-Gonnord M-F, Antonyuk LP, Colina M, Chernyshev AV, Frolov I, Ignatov VV. 1997a Spectroscopic characterization of the uptake of essential and xenobiotic metal cations in cells of the soil

bacterium *Azospirillum brasilense*. *Biochem Mol Biol Int* **41**, 123–130

Kamnev AA, Ristić M, Antonyuk LP, Chernyshev AV, Ignatov VV. 1997b Fourier transform infrared spectroscopic study of intact cells of the nitrogen-fixing bacterium *Azospirillum brasilense*. *J Mol Struct* **408/409**, 201–205

Kamnev AA, Ristić M, Colina M, Antonyuk LP, Chernyshev AV, Ignatov VV. 1997c Comparative spectroscopic study of intact cells and cell membranes of the bacterium *Azospirillum brasilense*. In: Carmona P., Navarro R, Hernanz A, eds. Spectroscopy of Biological Molecules: Modern Trends. Dordrecht: Kluwer Academic Publishers: 313–314.

Lehninger AV. 1993 Principles of Biochemistry, New York: North Publishers.

Magasanik B, Neidhardt FC. 1987. Regulation of nitrogen and carbon utilization. In: Neidhardt FC, Ingraham JL, Brooks L, Magasanik B, Schaechter M, Umbarger HE, eds. *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. Washington, DC: ASM: 1318–1325.

Meister A. 1980 Catalytic mechanism of glutamine synthetase; overview of glutamine metabolism. In: Mora J, Palacios R, eds. Glutamine: Metabolism, Enzymology, and Regulation. New York: Academic Press, 1–40.

Pirola MC, Monopoli R, Aliverti A, Zanetti G. 1992 Isolation and characterization of glutamine synthetase from diazotroph *Azospirillum brasilense*. *Int J Biochem* **24**, 1749–1754

Pushkin AV. 1990 Glutamine synthetase of plants and microorganisms. In: Poglazov BF, ed., Advances in Biological Chemistry , V. 31. Moscow: Nauka: 25–49. (in Rus.)

Segal A, Stadtman ER. 1972 Variation of the conformational states of *Escherichia coli* glutamine synthetase by interaction with different divalent cations. *Arch Biochem Biophys* **152**, 367–377

Shapiro BM, Stadtman ER. 1970 Glutamine synthetase (*Escherichia coli*) In: Tabor H, Tabor CW, eds. Methods in Enzymology, Vol. 17, New York: Academic Press, 910–922.

Stadtman ER, Mura U, Chock PB, Rhee SG. 1980 The interconvertible enzyme cascade that regulates glutamine synthetase activity. In: Mora J, Palacios R, eds. Glutamine: Metabolism, Enzymology, and Regulation. New York: Academic Press: 41–59.

Westby CA, Enderlin CS, Steinberg NA, Joseph CM, Meeks JC. 1987 Assimilation of $^{13}NH_4$ by *Azospirillum brasilense* grown under nitrogen limitation and excess. *J Bacteriol* **169**, 4211–4214.

Zhang Y, Burris RH, Ludden PW, Roberts GP. 1997 Regulation of nitrogen fixation in *Azospirillum brasilense*. *FEMS Microbiol Lett* **152**, 195–204

Zhulin IB, Bespalov VA, Johnson MS, Taylor BL. 1996 Oxygen taxis and proton motive force in *Azospirillum brasilense*. *J Bacteriol* **178**, 5199–5204