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Influence of divalent cations on the catalytic properties and secondary structure of unadenylylated glutamine synthetase from *Azospirillum brasilense*

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Abstract

Fully unadenylylated glutamine synthetase (GS) from the endophytic bacterium *Azospirillum brasilense* Sp245 was isolated and purified. The enzyme was electrophoretically homogeneous and contained strongly bound metal ions, which could not be removed by dialysis. Mn^{2+}, Mg^{2+}, and Co^{2+} were found to be effective in supporting biosynthetic activity of the *A. brasilense* GS. Some kinetic properties of Mn^{2+}-activated and Mg^{2+}-activated unadenylylated GS were characterized. Circular dichroism analysis of the enzyme showed that the *A. brasilense* GS is a highly structured protein: 59% of its residues form α-helices and 13% β-strands. Removal of the metal ions from the *A. brasilense* GS by treatment with EDTA resulted in alterations in the enzyme secondary structure.

Abbreviations: GS – glutamine synthetase; GltS – glutamate synthase; CD – circular dichroism.

Introduction

Glutamine synthetase (EC 6.3.1.2.; GS) catalyzes the ATP-dependent and metal-ion dependent synthesis of L-glutamine from L-glutamate and ammonia. It is well documented that GS, together with glutamate synthase (GltS), forms the main pathway for ammonia assimilation in bacteria (Westby *et al*. 1987; Vanoni & Curti 1999). In addition to its assimilatory function, GS is involved in metabolic regulation and it is recognized as a key enzyme of nitrogen metabolism in many organisms including bacteria. Regulation of activity and synthesis of bacterial GSs is very complex and has so far been investigated in detail for enteric bacteria only. GS activity in many bacteria, including *Azospirillum brasilense*, is modulated by reversible adenylylation in response to the cellular N-status. The enzyme is maintained in a top-active unadenylylated or slightly adenylylated form under nitrogen-limiting conditions, and the adenylylation level increases under the conditions of ammonia abundance (Segal & Stadtman 1972; Pirola *et al*. 1992).

Bacterial GS molecules are dodecamers formed from two face-to-face hexameric rings of subunits with 12 active sites formed between the monomers (Yamashita *et al*. 1989; Eisenberg *et al*. 2000). Divalent cations (commonly, Mg^{2+}, Mn^{2+}, or Co^{2+}) are absolutely necessary for the activity of all known bac-

terial GSs both in the case of glutamine synthesis and for the transferase reaction catalyzed by the enzyme. The X-ray crystallographic study of the *Salmonella typhimurium* GS revealed that each active site of the enzyme has two divalent cation binding sites, n1 and n2 (Yamashita *et al*. 1989); both of them must be saturated for the activity to be expressed. Judging from the earlier data obtained with $^{54}Mn^{2+}$ (Denton & Ginsburg 1969), along with the n1 and n2 sites, GS of enteric bacteria has 48 additional metal binding sites per oligomer. These additional cation binding sites have relatively low affinity (Denton & Ginsburg 1969); they are considered to be arranged outside the active sites of the enzyme and to be important for the conformational stability of the molecule.

Despite the similarity of the studied bacterial GSs in their molecular weights and quarternary structures, the enzymes from different bacteria differ in their divalent cation specificity. Thus, the *Escherichia coli* GS and *Mycobacterium smegmatis* GS are similar in their molecular organization; they both are also subject to regulation by adenylylation-deadenylylation (Matsuoka & Kimura 1985; Eisenberg *et al*. 2000). However, the metal specificity of the enzymes is different, and in the case of the *M. smegmatis* GS it is not altered by adenylylation of the enzyme (Matsuoka & Kimura 1985).

A. brasilense is a nitrogen-fixing plant-associated and plant growth-promoting bacterium (Döbereiner & Pedrosa 1987; Schloter & Hartmann 1998). Among several hundred *A. brasilense* strains described to date, strain Sp245 is unique in its ability to penetrate inside the wheat root and to form a strong intraroot and intracellular population (about 10^5 cells per gram of roots) existing within the whole vegetation period (Schloter & Hartmann 1998). Nitrogen metabolism of this bacterium may be supposed to have some specific features, since strain Sp245 as a simbiont successfully meets the needs of the host plant in bound nitrogen (Döbereiner & Pedrosa 1987).

Nitrogen metabolism of *A. brasilense* has still not been adequately explored. As for the enzymes of ammonia assimilation, only GltS of *A. brasilense* is relatively well studied (see Vanoni & Curti 1999 and references therein). Previous research from our laboratories gave the first data on the enzymology of the *A. brasilense* GS (Pirola *et al*. 1992; Bespalova *et al*. 1994, 1999). In particular, 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), and the kinetic behavior of the enzyme in an average state of adenylylation was characterized. The kinetic behavior appeared to depend strongly on the nature of the divalent cation used for GS activation (Bespalova *et al*. 1999).

Since divalent cation specificity of many bacterial GSs depends on their state of adenylylation, it was reasonable to isolate and purify unadenylylated GS from *A. brasilense* and to investigate the cation specificity of the enzyme in order to compare the properties of the unadenylylated and adenylylated forms. Another goal was to study the secondary structure of the *A. brasilense* GS and its possible dependence on such native components of GS as divalent cations.

As a further step in our study of the *A. brasilense* GS, in the present work we obtained a fully unadenylylated form of this enzyme and investigated its metal specificity and some kinetic properties. Circular dichroism (CD) spectroscopic data on the secondary structure of the unadenylylated *A. brasilense* GS and the influence of divalent cations on the secondary structure are also presented and dicussed.

Materials and methods

Growth of Azospirillum brasilense Sp245

A. brasilense Sp245 was kindly provided by Prof. J. Vanderleyden (F.A. Janssens Laboratory of Genetics, KU Leuven, Belgium) and grown in a minimal salt medium (Ratti *et al*. 1985) supplemented with 0.5% (w/v) sodium succinate and 5 mM L-glutamate at 30 °C under shaking. Cells grown to the late exponential phase were harvested and kept frozen at −20 °C until they were used for GS purification.

Purification of the Azospirillum brasilense Sp245 GS

Frozen cells were suspended in an extraction buffer (20 mM imidazole/HCl, 1 mM $MnCl_2$, pH 7.5) and disrupted by passing through a French press at 60 MPa. DNAse I (Sigma) was added to the crude extract to decrease viscosity (up to a final concentration of 1 μg/ml). After a 30-min incubation on ice the homogenate was centrifuged, and GS was precipitated between 35% and 70% saturation with ammonium sulfate. The precipitated protein was dissolved in 20 mM Hepes/NaOH buffer, supplemented with 1 mM $MnCl_2$, pH 7.5, and dialyzed overnight against 2000 volumes of the same buffer. The sample

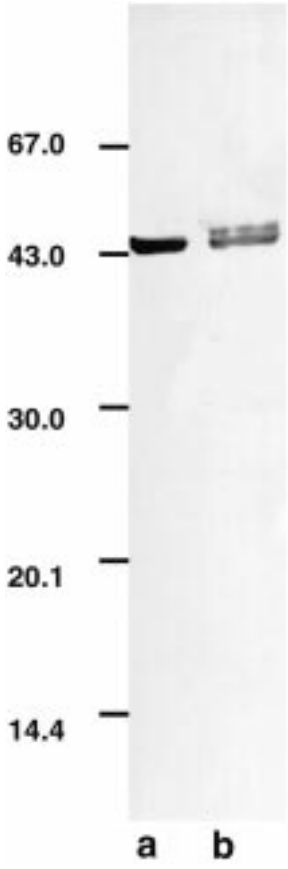

Fig. 1. Estimation of homogeneity and subunits composition of unadenylylated glutamine synthetase (GS) from *Azospirillum brasilense* Sp245 by SDS/gel electrophoresis (a). The slightly adenylylated GS from *A. brasilense* Sp245 (b) is presented for comparison. Positions of marker with molecular masses in kDa are indicated on the left. Markers: albumin (67.0), ovalbumin (43.0), carbonic anhydrase (30.0), trypsin inhibitor (20.1) and α-lactalbumin (14.4).

was then subjected to two sequential FPLC chromatographic runs, first on a Q-Sepharose HP column (Pharmacia Biotech) equilibrated in 50 mM Tris-HCl buffer, pH 8.0, and then on a Mono Q column (Pharmacia Biotech) equilibrated in 20 mM Tris-HCl buffer, pH 7.5. In both cases, for eluting the GS, a linear gradient from 0 to 1 M NaCl in the equilibrated buffer was used.

After the second chromatographic run, the fraction showing GS activity was passed through a Sephadex G-25 column equilibrated with 20 mM imidazole/HCl buffer (pH 6.3) in order to desalt the preparation, and applied to an Affi-gel Blue column (Bio-Rad) equilibrated in 20 mM imidazole/HCl buffer (pH 6.3) containing 1 mM $MnCl_2$. The Affi-gel Blue column was then washed with five volumes of the same buffer containing 1 M NaCl, and selective elution of GS using the buffer containing 5 mM ADP was performed. Prior to use in further experiments, the enzyme solution was concentrated over an intensive air flow and dialyzed against 200 volumes of 20 mM Tris-HCl buffer (pH 7.0) for 24 h at 4 °C.

Preparation of metal ion-free GS

The *Azospirillum brasilense* Sp245 GS, purified as described above, contained divalent metal cations (see Results) and is referred to as the 'native GS'. To obtain metal-free GS, EDTA was added up to the final concentration of 5 mM to *A. brasilense* Sp245 GS solution, and after a 30-min incubation period the preparation was dialyzed against 200 volumes of 50 mM Tris-HCl buffer, pH 7.0 (for 24 h at 4 °C), to remove the EDTA together with the chelated cations.

Estimation of GS homogeneity and its subunits composition

For the estimation of homogeneity of the *A. brasilense* Sp245 GS and its subunits composition, SDS/PAGE (Laemmli 1970) was used. The dialyzed GS preparation was mixed with sample buffer, loaded onto a 12% (w/v) acrylamide gel and run at a constant voltage of 200 V. The gel was stained with silver nitrate (Sambrook *et al*. 1989).

Enzyme activity assays

In order to determine the GS activity, two methods were used. The transferase reaction was assayed by measuring the formation of γ-glutamylhydroxamate as described previously (Bespalova *et al.* 1999), which was used during isolation and purification of the enzyme. The biosynthetic reaction was assayed by measuring the formation of inorganic phosphate. The method was used in the modification described previously (Bespalova *et al.* 1999). In all cases, assay mixtures contained 100 mM Tris-HCl buffer, and their pH values were equal to 7.1. The substrate concentrations varied in different experiments; their exact values are given in the legend to Figure 2 and in the footnote to Table 1. The reaction was initiated by adding 50 μl of the enzyme preparation (final volume of the assay mixture 250 μl) and allowed to proceed for 15 min at 37°C. The reaction was stopped by adding 600 μl 1% $FeSO_4 \times 7H_2O$ in 0.3 N H_2SO_4 and 100 μl 6.6% $(NH_4)_2Mo_7O_{24} \times 4H_2O$ in 7.5 N H_2SO_4. The

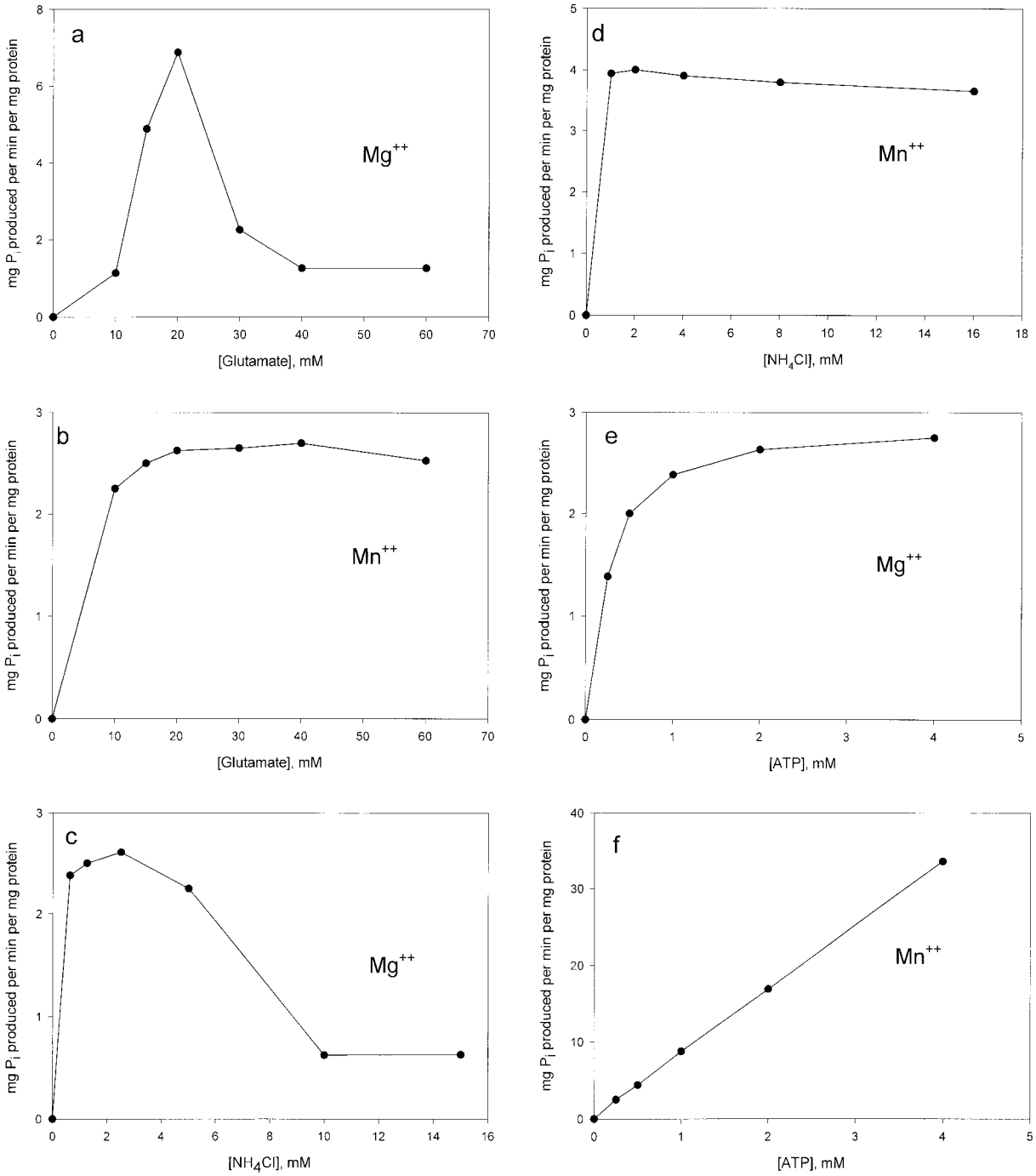

Fig. 2. Substrate saturation curves (a, b – L-glutamate; c, d – ammonium; e, f – ATP) for glutamine synthetase from *Azospirillum brasilense* Sp245 studied with Mg^{2+} (a, c, e) and Mn^{2+} (b, d, f). The assay mixtures contained 6 mM $MgCl_2$ (a, c, e) or $MnCl_2$ (b, d, f). Substrates and their concentrations were used as follows: for curves a, b – 1 mM ATP and 10 mM NH_4Cl; for curves c, d – 1 mM ATP and 20 mM L-glutamate (Na^+ form); for curves e, f – 10 mM NH_4Cl and 20 mM L-glutamate (Na^+ form).

Table 1. Biosynthetic activity* of the native and metal-free glutamine synthetase (GS) from *Azospirillum brasilense* Sp245

Metals in the assay mixture, 1 mM	Biosynthetic activity (mg P_i per min per mg protein)	
	Native GS	Metal-free GS
–	9.5	0
Mn^{2+}	11.0	6.9
Mg^{2+}	24.5	8.5
Co^{2+}	24.5	19.1

*Assay mixture contained 0.5 mM ATP, 10 mM NH_4Cl, and 20 mM L-glutamate.

coloration intensity was measured on a Specol 221 spectrophotometer at 700 nm.

The protein content was determined by the biuret method and by the Pierce Coomassie Protein Assay Reagent (Bradford 1976).

Circular dichroism (CD) measurements

CD spectra were recorded on a JASCO J-500C spectropolarimeter (Japan) using a solution with a protein concentration of 0.8 mg/ml. The results were expressed as molar ellipticity, $[\theta]$ (deg$\times$cm$^2\times$d mol^{-1}), based on a mean amino acid residue weight (MRW) assuming its average weight to be equal to 115 Da. The molar ellipticity was determined as $[\theta]_\lambda = \theta \times 100(\mathrm{MRW})/cl$, where c is the protein concentration in mg/ml, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at a wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg $\times$ cm^2 $\times$ dmol^{-1} and with JASCO standard non-hygroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7910$ deg $\times$ cm^2 $\times$ dmol^{-1}. Calculation of the content of the GS secondary structure elements was performed using the CONTIN program (Provencher 1982).

Results

The unadenylylated GS from Azospirillum brasilense Sp245. Native and metal ion-free GS

For strain Sp245, in the present work we used the growth conditions which had earlier been used for *A. brasilense* Sp6 (Pirola *et al.* 1992), and also introduced some modifications in the enzyme purification procedures developed earlier for strain Sp6 (Pirola *et al.* 1992) and strain Sp245 (Bespalova *et al.* 1994). This allowed us to obtain the fully unadenylylated form of GS from *A. brasilense* Sp245. The preparation obtained was electrophoretically homogeneous (Figure 1a). As shown earlier (Pirola *et al.* 1992), the unadenylylated and adenylylated subunits of the *A. brasilense* GS separate during electrophoresis in the presence of SDS. The GS preparation obtained as described in the Materials and Methods section contained a single type of subunits, *viz.*, unadenylylated, with the higher electrophoretic mobility. For comparison, we also present the electrophoregram of a slightly adenylylated GS preparation from *A. brasilense* Sp245 obtained by us (Figure 1b), which contains both types of subunits. The bacteria used for obtaining the latter GS sample were grown in a minimal salt medium supplemented with malate (0.5%, w/v) as the carbon source and ammonium sulfate (5 mM) as the nitrogen source.

The homogeneous unadenylylated GS from *A. brasilense* Sp245 contained divalent metal cations, and dialysis (the final purification step) failed to remove them from the enzyme. This conclusion is evident from the fact that the enzyme exhibited activity in the biosynthetic reaction when divalent cations in the assay mixture were omitted (Table 1). The GS preparation obtained was referred to as the 'native GS'. In order to remove divalent metal cations from the enzyme, we used the procedure described for the *E. coli* GS (Shapiro & Ginsburg 1968): the GS preparation was treated with EDTA which was then removed by dialysis (see Materials and methods for details). After such a treatment, the *A. brasilense* Sp245 GS was inactive in the absence of divalent cations in the assay mixture (Table 1). The results obtained suggest that the native unadenylylated GS contains metal ions at their active sites. It is significant to note that the native GS was more stable than the metal ion-free enzyme. The former was kept at 4 °C without any loss of the

biosynthetic activity for at least 3 months, whereas under the same storage conditions the activity of the latter decreased *ca.* 1.5-fold in a week.

Efficiency of divalent cations in supporting the GS activity

All the three tested cations, Mn^{2+}, Mg^{2+}, and Co^{2+}, were effective in supporting biosynthetic activity of the unadenylylated GS from *A. brasilense* Sp245, both with the native GS and with the metal ion-free enzyme (Table 1). It is essential that in all cases the native GS was more active (Table 1, left column) than the metal ion-free GS (Table 1, right column). The native *A. brasilense* Sp245 GS exhibited maximal activity with Mg^{2+} and Co^{2+} (the values were equal), whereas Mn^{2+} was about two times less effective. The cobaltous ion was also most effective in supporting biosynthetic activity of the metal-ion free enzyme (Table 1, right column), Mn^{2+} and Mg^{2+} being less effective. For both the native and metal ion-free enzyme, Mn^{2+} was the least effective.

Some kinetic properties of Mn^{2+}-activated and Mg^{2+}-activated unadenylylated GS

Kinetic properties of the unadenylylated GS in the biosynthetic reaction were studied in the presence of Mn^{2+} or Mg^{2+} as activating cations. For kinetic studies of the Mn^{2+}-activated and Mg^{2+}-activated GS of *A. brasilense* Sp245, we were forced to use the metal ion-free enzyme since, as can be seen from the experimental data presented above, the enzyme preparation obtained in the course of purification contained cations at the active centers. Similar to the GS from *A. brasilense* Sp245 in an average adenylylation state (Bespalova *et al.* 1999), for the unadenylylated Mg^{2+}-activated GS from this strain of *A. brasilense* substrate inhibition was characteristic (Figure 2, curves a and c). However, it should be emphasized that the inhibition was significantly more pronounced in the case of the unadenylylated form (82% in the case of glutamate and 76% in the case of ammonium) as compared to the GS in an average adenylylation state. The latter *A. brasilense* Sp245 GS form showed 35% and 28% inhibition, respectively (Bespalova *et al.* 1999).

Substitution of manganese for Mg^{2+} as the activating cation altered the substrate saturation kinetics of the unadenylylated *A. brasilense* Sp245 GS (Figure 2, curves b and d). While substrate inhibition still did take place, it was rather weak and did not exceed 7% and 6% for glutamate and ammonium, respectively. Comparison of these data to the analogous data obtained earlier for the GS in an average adenylylation state (Bespalova *et al.* 1999) shows that adenylylation-deadenylylation drastically changes the kinetic behavior of the *A. brasilense* Sp245 GS with Mn^{2+} as the activating cation. For instance, in the case of unadenylylated GS (see Figure 2, curve d) saturation occurs already at 2 mM ammonium, and higher concentrations of this substrate lead to a decrease in the reaction rate. On the contrary, in the case of the GS in an average state of adenylylation, substrate saturation was still not observed even at 60 to 70 mM ammonium (Bespalova *et al.* 1999).

The kinetic behavior of Mg^{2+}-activated and Mn^{2+}-activated unadenylylated GS from *A. brasilense* Sp245 towards the third substrate, ATP, was different as well (Figure 2, curves e and f). In the ATP concentration range 0.25 to 4 mM, the reaction rate was strictly directly proportional to the ATP concentration for the Mn^{2+}-activated GS (Figure 2, curve f), whereas it exhibited a hyperbolic dependence for the Mg^{2+}-activated GS (Figure 2, curve e).

To conclude, for the unadenylylated *A. brasilense* GS, both Mg^{2+}- and Mn^{2+}-activated, a complex kinetic behavior is observed which does not follow the Michaelis-Menten kinetics. Similar to the GS in an average adenylylation state (Bespalova *et al.* 1999), exchange of the activating cation altered the kinetic behavior of the enzyme.

Circular dichroism study of the unadenylylated GS

For CD spectroscopic studies of the unadenylylated form of *A. brasilense* Sp245 GS, the preparation of the native enzyme was used. As was mentioned above, this preparation contained cations bound to the protein. CD spectra of the native GS, to which 1 mM Mg^{2+}, Mg^{2+}, Co^{2+}, or EDTA was added, were also measured. Besides that, a CD spectrum of the native GS after treatment with 5 mM EDTA (which, as is seen from the data presented above, removes the cations from the binding sites) was recorded. The spectra obtained were used for calculating the content of secondary structure elements of the enzyme (Table 2). The first of the spectra mentioned (for the native GS) and the last one (metal-free GS) are shown in Figure 3.

The general shape of the CD spectrum for the native unadenylylated *A. brasilense* Sp245 GS (Figure 3, dotted line) is somewhat different from those reported for a number of other bacterial GSs (Hunt &

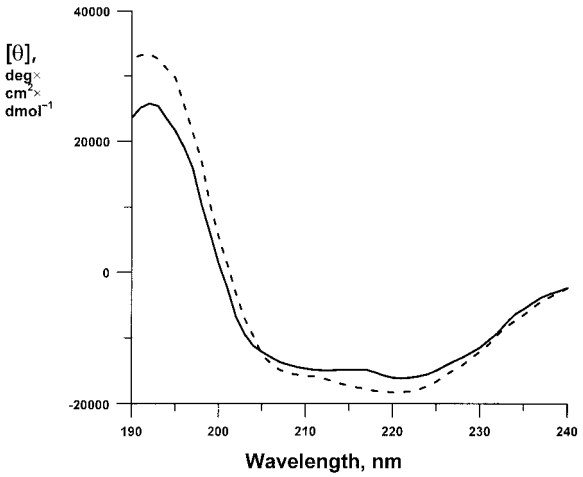

Fig. 3. Circular dichroism spectra of the native glutamine synthetase (dotted line) and the metal-free glutamine synthetase (solid line) from *Azospirillum brasilense* Sp245. Spectra were obtained at 25 °C as described in the Materials and methods section.

Table 2. Content of the secondary structure elements of the *Azospirillum brasilense* Sp245 glutamine synthetase (GS) calculated from circular dichroism (CD) spectra

Variants of treatment	Secondary structure elements, %		
	α-helices	β-strands	Unordered structure
GS*	59 ± 2	13 ± 5	28 ± 3
GS + 1 mM $CoCl_2$**	57 ± 2	21 ± 4	22 ± 4
GS + 1 mM $MgCl_2$	52 ± 4	21 ± 7	27 ± 7
GS + 1 mM $MnCl_2$	56 ± 2	13 ± 4	31 ± 3
GS + 1 mM EDTA	43 ± 1	24 ± 3	32 ± 2
GS + 5 mM EDTA	38 ± 2	32 ± 4	30 ± 3

* The native enzyme was used.
**The native enzyme was incubated with $CoCl_2$ (or $MgCl_2$, or $MnCl_2$, or EDTA) for 30 min and then CD spectra were recorded.

Ginsburg 1972; Matsuoka & Kimura 1985; Kimura & Sugano 1992). This may probably be connected with differences in the amino acid sequences of GSs obtained from different sources (Colombo & Villafranca 1986; Bozouklian & Elmerich 1986; Eisenberg *et al.* 2000) and the resulting differences in their secondary structures. Adding divalent metal cations to the native enzyme caused relatively small alterations in the CD spectrum of *A. brasilense* Sp245 GS (not shown). On the contrary, a 30-min incubation of the *A. brasilense* Sp245 GS with 5 mM EDTA leading to the removal of the cations from the protein molecule caused some noticeable changes in the spectrum (Figure 3, solid line). As can be seen comparing the two spectra, the removal of the cations led to a decrease in molar ellipticity in the wavelength range of 190–205 nm and some increase in the range of 205–230 nm.

As is seen from the results of calculations based on the CD spectra using the CONTIN program (Table 2), the native unadenylylated *A. brasilense* Sp245 GS (bearing bound metal cations, as was determined in the experiments described above) contains a high proportion of α-helix (59% of the enzyme polypeptide chain). The proportion of β-strands appeared to be lower (13%). As a whole, the native unadenylylated *A. brasilense* Sp245 GS appears to be a highly structured protein: according to the calculations, 72% of the molecule is structured (α-helices and β-strands) while only 28% is not structured. As follows from the data presented in Table 2, introducing additional divalent cations to the system (besides those already bound to the protein) did not cause significant changes in the content of the secondary structure elements of the protein. Conversely, the removal of the metal ions resulted in alterations in the enzyme secondary structure. Calculations showed that the proportion of α-helix in the metal ion-free enzyme is significantly diminished as compared to that in the native enzyme, whereas the proportion of β-strands is increased (see Table 2). In general, basing on the CD spectroscopic data it can be concluded that the metal ion-free *A. brasilense* Sp245 GS remains a highly structured protein: according to the calculations, about 70% of the molecule is structured and only ca. 30% is not structured (Table 2).

Discussion

In the course of this work we succeeded in obtaining an *A. brasilense* Sp245 GS preparation which contained only one type of subunits, *viz.*, completely unadenylylated GS. As a rule, it has not been possible to obtain bacterial GS in extreme states of adenylylation (i.e., E_0 or E_{12}), and in order to obtain and study completely unadenylylated GS, either mutant strains are used (Eisenberg *et al.* 2000), or partly adenylylated GSs are treated with snake venom phosphodiesterase for splitting off the AMP moieties (Matsuoka & Kimura 1985; Pirola *et al.* 1992). The fact that we succeeded in obtaining the unadenylylated GS is most likely related to the specificity of the *A. brasilense* strain used. As noted above, strain Sp245 is unique in its ability to penetrate inside the wheat root and inside the root hair (Assmus *et al.*

1995; Schloter & Hartmann 1998). In contrast to many other *A. brasilense* strains, strain Sp245 has evolutionarily got adapted to existence under the conditions of endophytic symbiosis. It is reasonable to suggest that this endophytic bacterium has some specific features of its nitrogen metabolism, in particular in adenylylation-deadenylylation of GS. An indirect evidence for this suggestion is the fact observed in the course of the present work that using the identical protocol for growth conditions, cell disruption and primary purification steps (treatment with DNAse I, salting out, FPLC chromatography on a Q-Sepharose HP column; see Materials and methods) for strains Sp245 and Sp6 gave, in the case of Sp245, partly purified unadenylylated GS, whereas for Sp6 electrophoresis revealed two types of subunits, *viz.*, both adenylylated and unadenylylated.

As follows from the data presented here, the unadenylylated GS from *A. brasilense* Sp245 contains high affinity sites of metal binding: cations could not be removed by dialysis against a cation-free buffer. Metal cations are most probably bound at the active sites of the enzyme. This assumption is corroborated by the fact that the native *A. brasilense* Sp245 GS is active in the biosynthetic reaction when there were no divalent cations in the assay mixture, whereas the EDTA-treated GS is not. Two experimental facts reported here, first, that the native GS is more active in biosynthetic reaction than the metal ion-free enzyme and, second, that the native GS is significantly more stable during storage, provide evidence that in the case of the unadenylylated GS from *A. brasilense* Sp245, metal ions are involved not only in the catalysis, but in the maintenance of the active conformation as well. This finding is in good agreement with data on some other bacterial GSs (Shapiro & Ginsburg 1968; Denton & Ginsburg 1969; Hunt & Ginsburg 1972; Matsuoka & Kimura 1985). It remains unknown what kind of metal ion is bound to the native unadenylylated GS of *A. brasilense* Sp245. It can be reasoned that metal-binding sites of the enzyme are occupied with manganese cations, as 1 mM $MnCl_2$ had been used by us during purification of the enzyme (see Materials and methods); note that manganese is commonly used as a component of buffers applied for isolation and purification of bacterial GSs. It cannot be excluded that some other cations were bound to the unadenylylated GS of *A. brasilense* Sp245 as well.

The *A. brasilense* Sp245 GS appeared to be different from the *E. coli* GS in its specificity to divalent cations. For the *E. coli* GS, the specificity to divalent cations in the biosynthetic reaction is known to depend on its adenylylation state. The unadenylylated *E. coli* GS is active with Mg^{2+} and inactive with Mn^{2+}; upon adenylylation its activity with Mg^{2+} decreases, the enzyme becomes Mn^{2+}-specific, and with increasing adenylylation state its Mn^{2+}-supported activity grows while the Mg^{2+}-supported one diminishes (Shapiro & Ginsburg 1968; Segal & Stadtman 1972). Co^{2+}, similar to magnesium, supports the activity of the unadenylylated *E. coli* GS, but is inactive in the case of the adenylylated GS (Segal & Stadtman 1972). In the case of *A. brasilense* Sp245 GS, its specificity to divalent metal cations appears to be essentially different. Both the unadenylylated *A. brasilense* Sp245 GS (see Table 1) and its form in an average adenylylation state, as was shown earlier (Bespalova *et al.* 1999), are active with each of the three aforementioned cations. In general, the *A. brasilense* Sp245 GS metal specificity is independent from the adenylylation state of the enzyme, which, in particular, is also characteristic for the adenylylatable GS from *M. smegmatis* (Matsuoka & Kimura 1985). It is noteworthy, however, that at pH 7.1, which was shown to be the intracellular pH in *A. brasilense* (Zhulin *et al.* 1996), the efficiency of divalent cations in supporting the activity of the unadenylylated *A. brasilense* GS was different (see Table 1), whereas the GS in an average adenylylation state was equally active with each of the three cations (Bespalova *et al.* 1999). It should also be emphasized that, despite the similarity in metal specificity of unadenylylated and adenylylated forms of *A. brasilense* GS, differences in their kinetic behavior were rather significant.

It is noteworthy that the *A. brasilense* GS differs from the *E. coli* GS not only in divalent cation specificity but in the properties of metal ion-free form of the enzyme as well. Thus, in the case of *E. coli* GS, cation-free form is catalytically inactive (Shapiro & Ginsburg 1968), and in order to restore the activity of the enzyme, its preincubation with certain cations is necessary (for example, Ca^{2+}, Mn^{2+}, or Mg^{2+} can reactivate the *E. coli* GS, but Co^{2+} cannot). In contrast, in the case of the *A. brasilense* GS, its metal ion-free form is catalytically active without any preincubation (when Co^{2+}, Mn^{2+}, or Mg^{2+} is present in assay mixture, see Table 1).

The results obtained provide evidence that metal ions play a role also in maintaining the secondary structure of the unadenylylated *A. brasilense* GS. Thus, in the native enzyme, which is a metal-bearing protein, the proportion of α-helix in the secondary

structure was very high, while the removal of the cations was accompanied by a decrease in the proportion of α-helical configuration (from 59% to 38%). Another effect of metal ion removal was an increase in the proportion of β-strands. It is noteworthy that GSs from different bacteria essentially differ in the way by which metal ions influence the enzyme secondary structure. Thus, with the *E. coli* GS, removing or adding 1 mM of one of specific divalent cations, which markedly affected a number of enzyme properties, had no influence on the CD spectrum and the content of the secondary structure elements calculated from the spectrum (Hunt & Ginsburg 1972). CD analyses of the *M. smegmatis* GS revealed a change in the secondary structure on the addition of Mg^{2+}, suggesting that the content of the unstructured conformation decreased (Matsuoka & Kimura 1985). In the case of the *Bacillus stearothermophilus* GS, the native form of which is cation-free, addition of high concentrations of Mg^{2+} (10–50 mM) or Mn^{2+} (up to 10 mM) resulted in changes in CD spectra of the enzyme (Hachimori *et al.* 1974). According to estimations from the CD data, the metal-bearing *B. stearothermophilus* GS, similar to the metal-bearing *A. brasilense* GS, have a larger part of the α-helical configuration as compared to the metal-free enzyme (Hachimori *et al.* 1974).

In general, the *A. brasilense* Sp245 GS seems to have a more ordered structure as compared to other bacterial GSs studied so far. As mentioned above, 72% of the molecule of the native unadenylylated *A. brasilense* GS is structured (59% α-helical conformation and 13% β-strands). In the case of the *E. coli* GS, 36% of the amino acid residues in the molecule are in the form of α-helix and 26% as β-sheets (Colombo & Villafranca 1986, the values calculated from the amino acid sequence). The content of secondary structure elements calulated from CD spectra of the *E. coli* GS earlier was very close to the latter (36% protein as α-helix and 24% protein as β-pleated sheets, Hunt & Ginsburg 1972). The fractions of α-helix and β-structure of the native *B. stearothermophilus* GS as estimated from the CD data were 7 and 34%, respectively (Hachimori *et al.* 1974).

To conclude, the unadenylylated *A. brasilense* GS has a number of features which make it similar to other bacterial GSs. And, at the same time, it has some specific features. The unadenylylated GS is a highly structured protein and remains highly structured after the removal of the metal ions. The enzyme specificity to divalent metal cations is independent from the adenylylation state; however, the kinetic behavior of the GS forms, which differ in the adenylylation state, is different.

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