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Structural Characterization of Glutamine Synthetase from *Azospirillum brasilense*

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Abstract: CD spectroscopic study of the secondary structure of partly adenylylated glutamine synthetase (GS) of the bacterium *Azospirillum brasilense* showed both the native and cation-free (EDTA-treated) enzyme to be highly structured (58 and 49% as α -helices, 10 and 20% as β -structure, respectively). Mg^{2+} , Mn^{2+} , or Co^{2+} , when added to the native GS, had little effect on its CD spectrum, whereas their effects on the cation-free GS were more pronounced. Emission (^{57}Co) Mössbauer spectroscopic (EMS) study of $^{57}Co^{2+}$ -doped cation-free GS in frozen solution and in the dried state gave similar spectra and Mössbauer parameters for the corresponding spectral components, reflecting the ability of the Co^{2+} -enzyme complex to retain its properties upon

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drying. The EMS data show that (a) *A. brasilense* GS has 2 cation-binding sites per active center and (b) one site has a higher affinity to Co^{2+} than the other, in line with the data on other bacterial GSs. © 2004 Wiley Periodicals, Inc. *Biopolymers* 74: 64–68, 2004

Keywords: glutamine synthetase; *Azospirillum brasilense*; secondary structure; active center; CD; ^{57}Co emission Mössbauer spectroscopy

INTRODUCTION

In phytostimulating rhizobacteria of the genus *Azospirillum*,¹ nitrogen metabolism has not yet been fully explored; in *A. brasilense* Sp245, a facultative endophyte,² it may have some specific features.³

Glutamine synthetase (GS; EC 6.3.1.2.), a ubiquitous key enzyme of nitrogen metabolism,⁴ catalyzes the ATP- and metal ion-dependent synthesis of L-glutamine from L-glutamate and NH_4^+ . Regulation of activity and synthesis of bacterial GSs is very complex and has so far been studied in detail mostly for enteric bacteria.⁴ GS activity in many bacteria, including *Azospirillum brasilense*,⁵ is modulated in response to the cellular N-status by reversible adenylation. GS is maintained in a top-active unadenylated or slightly adenylylated form under N-limiting conditions; its adenylation state (ranging from E_0 up to E_{12} , with 12 adenylylatable subunits in the GS molecule⁴) increases at NH_4^+ abundance.^{5,6}

Our previous studies^{7,8} have shown that many properties of *A. brasilense* GS depend on the nature of the activating divalent cations, including their effects on the catalytic behavior and secondary structure of fully nonadenylated *A. brasilense* GS.⁸ This work on partly adenylylated GS from *A. brasilense* Sp245 was aimed at (a) comparing the effects of Mg^{2+} , Mn^{2+} , and Co^{2+} on its secondary structure using CD spectroscopy, and (b) probing Co^{2+} binding at its active centers using ^{57}Co emission Mössbauer spectroscopy (EMS).

MATERIALS AND METHODS

Azospirillum brasilense Sp245 was grown in a minimal salt medium supplemented with 5 mM NH_4Cl as a nitrogen source and 0.6% Na malate as a carbon source.⁷ Isolation and purification of partly adenylylated GS and estimation of its activity were performed as described earlier.⁷ The preparations obtained were electrophoretically homogeneous,⁸ contained divalent cations (see Results and Discussion) and are referred to as “native GS.” The adenylation state was estimated using the Sigma Gel program by scanning the GS electrophoregrams⁸ and estimating the relative contents of adenylylated and nonadenylated subunits; it was 5.3 for the samples ($E_{5.3}$) used for CD studies and 2.2 for EMS

measurements ($E_{2.2}$). CD measurements, data treatment, and calculation of the GS secondary structure elements content were described earlier.⁸

To obtain cation-free enzyme, native GS was treated with 5 mM EDTA for 30 min and dialyzed against 200 volumes of 50 mM Tris HCl buffer, pH 7.0 (for 24 h at 4°C), or for EMS, against 1000 volumes of 50 mM KCl (pH 7.0; 4°C), to remove EDTA–metal chelates.

For EMS, $^{57}\text{CoCl}_2$ (1 mCi) was added to 1 mL of the cation-free GS solution at a ratio of ca. 24 $^{57}\text{Co}^{2+}$ ions per enzyme molecule (see below), incubated for 1 h at 295 K, and rapidly frozen in liquid nitrogen. The dry sample was obtained by drying the solution in air. Spectra were obtained using a conventional constant-acceleration Mössbauer spectrometer (absorber $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$) with a PC-operated multichannel analyzer by placing the ^{57}Co -doped samples (source) in a cryostat with liquid nitrogen ($T = 80$ K). Standard statistical analysis enabled calculation of the isomer shift (δ ; vs $\alpha\text{-Fe}$), quadrupole splitting (Δ), linewidth (Γ_{exp}), and relative areas of spectral components (S_i).

RESULTS AND DISCUSSION

The partly adenylylated GS isolated from *A. brasilense* Sp245 was active in the biosynthetic reaction without divalent cations in the assay mixture (not shown); it was referred to as “native GS.”⁸ After treating the latter with 5 mM EDTA and dialysis, the resulting GS was inactive without divalent cations in the assay mixture, showing that native partly adenylylated GS contains divalent cations at its active centers. The partly adenylylated GS, both native and cation free, showed biosynthetic activity with each of the cations tested (Mg^{2+} , Mn^{2+} , Co^{2+}). This proves that divalent cations added to the cation-free GS bind at its active centers, which is prerequisite for the activity of all known GSs to be expressed.^{4,7–9}

Bacterial GS molecules are dodecamers with 12 active sites located between the subunits.⁴ X-ray crystallography of *Salmonella typhimurium* GS has shown that each active site has two divalent cation-binding sites, n1 and n2, with the affinity of n1 for metal ions 50 times that of n2 (Ref. 4 and refs. therein). Comparing the data of Refs. 4 and 9, it can be inferred that outside the active centers, *Escherichia coli* GS has 48 additional metal-binding sites per oligomer (with relatively low affinity) considered to be important for

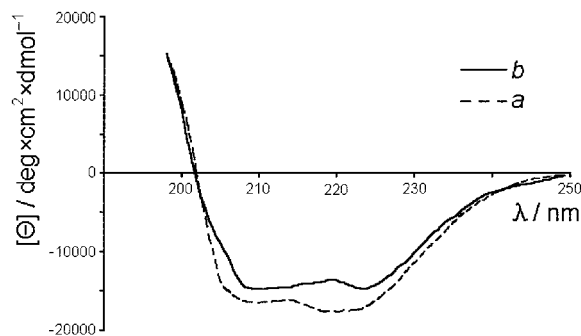


FIGURE 1 CD spectra of partly adenylylated (a) native and (b) cation-free (EDTA-treated) glutamine synthetase (GS; $E_{5,3}$) from *A. brasilense* Sp245.

conformational stability of the molecule, as well as an NH_4^+ -binding site.

Effects of Divalent Cations on the Secondary Structure of Partly Adenylylated GS

To study the effect of cations on the secondary structure of the partly adenylylated enzyme ($E_{5,3}$), CD spectra were measured for the native GS (Figure 1a), and for the latter with 1 mM Mg^{2+} , Mn^{2+} , or Co^{2+} added, which all had little effect on the shape of its CD spectrum (not shown). However, the spectrum of the native GS changed after treatment with 5 mM EDTA (Figure 1b) reflecting changes in its secondary structure (a negative maximum at 208 nm, with molar ellipticities $[\Theta] \approx -1.6 \cdot 10^4 \text{ deg} \times \text{cm}^2 \times \text{dmol}^{-1}$, and a shoulder at ca. 222 nm are typical of α -helical proteins, whereas a similarly intensive broad negative band at ca. 215 nm shows in proteins rich in β -structure).¹⁰ Adding 1 mM Mg^{2+} , Mn^{2+} , or Co^{2+} to the cation-free GS altered its CD spectrum (not shown), in contrast to the native enzyme, making it closer to that of the latter. Thus, cation binding partly restores the secondary structure of the enzyme. The CD spectra were used for calculating the content of GS secondary structure elements.

Both the native and cation-free partly adenylylated GSs appear to be highly structured ($58 \pm 2\%$ and $49 \pm 3\%$ of the polypeptide as α -helices, $10 \pm 2\%$ and $20 \pm 2\%$ as β -structure, respectively, with $32 \pm 2\%$ and $31 \pm 2\%$ unordered). Thus, removing cations from the native GS lowers the proportion of α -helices and increases that of β -structure, similar to *nonadenylylated* native and cation-free *A. brasilense* GS.⁸

Adding Mg^{2+} or Co^{2+} to the cation-free GS increased the content of α -helices up to $57 \pm 2\%$ or $58 \pm 2\%$, respectively (though insignificantly for

Mn^{2+} , up to $52 \pm 3\%$); the β -structure content in the cation-free GS decreased upon adding Mn^{2+} (down to $10 \pm 2\%$) or Co^{2+} ($16 \pm 2\%$), but insignificantly for Mg^{2+} ($19 \pm 2\%$). Similar structural changes upon cation binding were reported for *Bacillus stearothermophilus* GS.¹¹

Thus, *A. brasilense* GS appears to be most structured among all bacterial GSs known so far (Refs. 8, 11, and 12, and refs. therein): the total content of α -helices and β -structure was 62% for native *E. coli* GS and 41% for *B. stearothermophilus* GS,¹¹ whereas for native *A. brasilense* GS it was close to 70% (see above⁸). The partly adenylylated *A. brasilense* GS studied is mostly structured with Mg^{2+} and Co^{2+} (more than native or cation-free GS) and least structured with Mn^{2+} (less than native or cation-free GS).

EMS Study of ^{57}Co -Doped Partly Adenylylated GS

To avoid multiple $^{57}\text{Co}^{2+}$ binding beyond active centers, which would make spectra hardly interpretable, $^{57}\text{Co}^{2+}$ was added in an amount slightly below that necessary for saturating the 24 cation-binding sites per GS molecule.⁴ EMS spectra of $^{57}\text{Co}^{2+}$ -doped cation-free *A. brasilense* GS in frozen solution and in the dry state (Figure 2a,b) are similar, each containing two components corresponding to two high-spin nucleogenic $^{57}\text{Fe}^{\text{II}}$ forms (Table I) with different coordination. The δ , Δ , and Γ_{exp} values for the corresponding spectral components in samples 1 and 2 (Table I) are statistically indistinguishable, showing that the Co^{2+} -enzyme complex retains its properties upon drying.

The Fe^{II} form with a lower $\Delta = 2.3\text{--}2.4 \text{ mm/s}$ may correspond to the site with a higher symmetry. Note that for *S. typhimurium* GS, the cation in site n1 (with a higher affinity) is coordinated by 3 Glu residues (i.e., 3 carboxyls), whereas in site n2 by one His and two Glu residues (i.e., a N-donor atom of the His and two carboxyls), and this structure is conserved in different GSs⁴ [additional ligand(s) may be H_2O].¹³ Thus, for *A. brasilense* GS, the higher Δ value (ca. 3.0–3.1 mm/s; see Table I) may reflect a lower coordination symmetry owing to different donor atoms. Similar parameters were reported for Fe^{II} -DNA complex: $\delta = 1.07 \text{ mm/s}$, $\Delta = 2.98 \text{ mm/s}$ at 300 K (Ref. 14, p. 382). Note that the His heterocycle is similar to the 5-membered heterocycle in purine that, in neutral DNA solutions, may coordinate Fe^{II} at N7. This is also in line with the higher proportion of the Fe^{II} form with $\Delta = 2.3\text{--}2.4 \text{ mm/s}$ (see Table I), which in *A. brasilense* GS may thus correspond to site n1 (with a higher affinity to cations), the other Fe^{II} form corre-

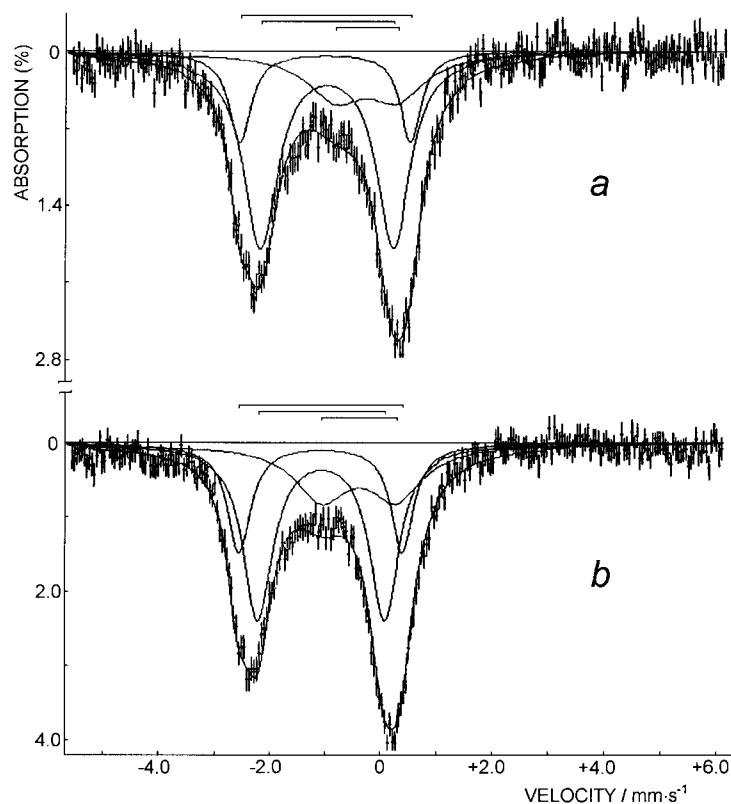


FIGURE 2 Emission Mössbauer spectra of $^{57}\text{Co}^{2+}$ -doped cation-free GS ($E_{2,2}$) from *A. brasilense* Sp245: (a) in frozen aqueous solution; (b) dry ($T = 80$ K).

sponding to site n2. Some differences in S_r for the corresponding forms in samples 1 and 2 may be due to nonequal changes in their Mössbauer factors in going from a frozen solution to a solid.^{14,15}

The yield of the stabilized $^{57}\text{Fe}^{\text{III}}$ components in samples 1 and 2, resulting from aftereffects of the $^{57}\text{Co} \rightarrow ^{57}\text{Fe}$ nuclear transformation^{15,16} (22–26%; see Table I) is much lower than for dilute rapidly frozen

Table I Mössbauer Parameters^a for $^{57}\text{Co}^{2+}$ -Doped Partly Adenylylated Glutamine Synthetase (GS, $E_{2,2}$) from *A. brasilense* Sp245 Calculated from Emission Mössbauer Spectra in Rapidly Frozen Aqueous Solution and in the Dry State ($T = 80$ K)

Sample	O/S ^b	δ^c (mm/s)	Δ^d (mm/s)	Γ_{exp}^e (mm/s)	S_r^f (%)	Fig.
1. Cation-free GS doped with $^{57}\text{Co}^{2+}$; frozen (solution)	+2	1.08 (0.02)	3.08 (0.08)	0.48 (0.05)	18 (1)	2a
	+2	1.05 (0.02)	2.39 (0.06)	0.75 (0.08)	60 (1)	
	+3	0.34 (0.10)	1.12 (0.20)	1.25 (0.30)	22 (1)	
2. Same as 1, dried (solid)	+2	1.08 (0.02)	2.97 (0.06)	0.52 (0.12)	24 (1)	2b
	+2	1.07 (0.02)	2.29 (0.06)	0.66 (0.08)	50 (1)	
	+3	0.39 (0.10)	1.37 (0.20)	1.19 (0.27)	26 (1)	

^a Errors given in parentheses.

^b Oxidation state of the stabilized nucleogenic ^{57}Fe form.

^c Isomer shift (vs α -Fe; converted to the absorption convention).

^d Quadrupole splitting.

^e Linewidth at half maximum.

^f Relative areas of the spectral components, representing relative contents of the corresponding nucleogenic Fe forms assuming a common recoilless fraction.

(80 K) aqueous $^{57}\text{CoCl}_2$ solutions,¹⁵ reflecting a firm binding of parent $^{57}\text{Co}^{\text{II}}$ in the intersubunit spaces of the GS oligomer⁴ to the organic ligands with higher electron–donor properties.

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