PURIFICATION AND CHARACTERIZATION OF GLUTAMINE SYNTHETASE FROM ALKALOPHILIC *BACILLUS* No. 170

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Glutamine synthetase from an alkalophilic *Bacillus* No. 170 was purified to homogeneity from cells grown in synthetic medium at pH 9.0. The purified enzyme had a molecular weight of 600,000 and a subunit size of 50,000, similar to the enzymes from *Bacillus subtilis* and *Bacillus cereus*. Either Mg^{2+} or Mn^{2+} activated the enzyme; however, the highest activity was obtained when 20 mM Mg^{2+} with 0.2 mM Mn^{2+} were added to the assay mixture. The peculiar divalent cation dependency of the glutamine synthetase was similar to that of other *Bacillus* enzymes but differed from that of the enteric bacteria enzymes. The kinetic properties of the enzyme, such as optimum pH, K_m for each substrate, V_{max} values, and inhibition by glutamine, were similar to those in the case of other *Bacillus* enzymes. The enzyme from this alkalophilic *Bacillus* was modified by iodoacetoamide or the ATP analog, 5'-*p*-fluorosulfonyl benzoyladenine (FSBA). Only Mg^{2+} -dependent activity was decreased by iodoacetamide, whereas each of the Mn^{2+} -, Mg^{2+} -, and Mg^{2+} plus Mn^{2+} -dependent activities was decreased with FSBA. The sequence of the NH_2 -terminal 20 amino acids of alkalophilic *Bacillus* glutamine synthetase was similar to those of other *Bacillus* enzymes.

Glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming) EC 6.3.1.2] is an important enzyme in nitrogen metabolism and ammonia assimilation in many prokaryotes, eukaryotes, and archaeobacteria. Consequently, the enzyme is subject to a variety of control mechanisms, and its regulation has been extensively studied in *Escherichia coli* and some enteric bacteria (11, 21, 23). In these bacteria,

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the enzymatic activity is regulated by a cascade of reactions culminating in either adenylylation or deadenylylation of each dodecameric subunit of the enzyme, depending upon the state of nitrogen availability (5,14). In contrast, there is no evidence that the *Bacillus subtilis* glutamine synthetase is regulated by reversible adenylylation although it appears that the mechanisms involved are different from those in *E. coli* and other enterics. In earlier papers, we reported the isolation and characterization of the glutamine synthetase from both *Bacillus cereus* and *Bacillus subtilis* (12,13), the putative amino acid sequence from the DNA sequence of each gene (16,18), and the determination of the substrate binding sites by chemical modification (15,17,24). To confirm the unique characteristic of the glutamine synthetase from these spore-forming bacteria, we have been investigating nitrogen metabolism in alkalophilic *Bacillus*. To understand the physiology of growth and the nitrogen metabolism in the alkalophilic *Bacillus*, we purified glutamine synthetase from cells grown at pH 9.0. We report here that the glutamine synthetase isolated from alkalophilic *Bacillus* No. 170 closely resembles those of other *Bacillus* enzymes in its catalytic properties and N-terminal amino acid sequence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Alkalophilic *Bacillus* No. 170 was grown aerobically to the early stationary phase at 37°C in modified alkaline medium (pH 9.0) (8) with glucose (11 mM)-glutamate (30 mM)-glycine (1.3 mM) as the carbon and nitrogen sources.

Assay method. Glutamine synthetase activity was measured by the biosynthetic or γ -glutamyltransferase assays as described previously (13,17). The standard biosynthetic assay mixture, in a final volume of 1.0 ml, contained 100 mM imidazole-HCl buffer, 5 mM ATP, 100 mM L-glutamate, 10 mM ammonia, divalent cation, and sufficient enzyme. As the divalent cations, 5 mM MnCl_2 , 20 mM MgCl_2 , or 0.2 mM MnCl_2 plus 20 mM MgCl_2 were added for assay of Mn^{2+} -, Mg^{2+} -, and Mg^{2+} plus Mn^{2+} -dependent activity, respectively. The pH of the buffer was 8.0 for Mg^{2+} -, 7.85 for Mg^{2+} plus Mn^{2+} -, and 6.4 for Mn^{2+} -dependent assay. Each reaction mixture was incubated at 37°C for 10 min. Specific activities are expressed as μmol of phosphate formed per minute per milligram protein. Protein in crude extracts was measured by the biuret method with bovine serum albumin as a standard. With the pure enzyme, the protein was determined either by the method of Lowry or from the absorbance at 280 nm. Preparation of rabbit antisera for *Bacillus subtilis* glutamine synthetase was described previously (13). Each glutamine synthetase from *B. subtilis* or *Escherichia coli* was purified from *E. coli* YMC11 harboring pSGS2 (9) or pEGS1. Each plasmid contained the *glnA* gene of *B. subtilis* or *E. coli*, respectively.

Purification of glutamine synthetase from alkalophilic *Bacillus* No. 170. Glutamine synthetase was purified from cells grown at pH 9.0, according to the modified method previously described in *Bacillus cereus* enzyme (13). Cells (14 g

wet weight) were suspended in four times their volume of buffer containing 50 mM Tris-HCl (pH 7.2), 0.1 mM EDTA, and 1 mM MnCl_2 . After rupture by sonication, crude extract was prepared by centrifugation at $20,000 \times g$ for 30 min. Streptomycin solution was added to the extract to a final concentration of 1%. After 30 min, the precipitate was removed by centrifugation at $20,000 \times g$ for 20 min. Ammonium sulfate was added to the supernatant to a final concentration of 40% saturation. The mixture was centrifuged and ammonium sulfate was further added to the supernatant to a final 65% saturation. The precipitate was dissolved in 5 ml of starting buffer, and this solution was applied to a column (3×50 cm) of Bio-Gel A-1.5 m, and eluted with 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM MnCl_2 . Fractions of 10 ml were collected. The fractions having glutamine synthetase activity (No. 21 to No. 23) were applied to a TSK gel DEAE-5PW column (0.75×7.5 cm, Tosoh, Tokyo, Japan) pre-equilibrated in the same buffer. The column was washed with a buffer containing 0.2 M KCl and developed with a linear 0.2–0.5 M KCl gradient. The four-step purification of glutamine synthetase from 14 g of wet cells resulted in 75-fold purification, with a yield of about 21%. The purification of the enzyme by selective affinity chromatography with 5'-AMP Sepharose and Affi-gel blue, as described for the isolation of some other bacterial glutamine synthetase, was not suitable for alkalophilic *Bacillus* enzyme.

RESULTS AND DISCUSSION

Homogeneity and subunit molecular weight

The purified enzyme was subjected to SDS-PAGE. As shown in Fig. 1, a single band (lane 2) appeared upon staining with Coomassie brilliant blue. The subunit molecular weight of the alkalophilic *Bacillus* No. 170 was 50,000, the same value as that of the enzyme from *B. subtilis* (lane 1); however, it was smaller than the 52,000 *E. coli* enzyme (lane 3). The results shown in Fig. 1 are in good agreement with those of *B. subtilis* glutamine synthetase which was 444 amino acids long (50,205 Da), compared with 469 for *E. coli* (51,823 Da) deduced from the nucleotide sequence of the cloned gene (18, 22). The molecular weight of alkalophilic *Bacillus* No. 170 glutamine synthetase was estimated to be 600,000 by gel filtration on Bio Gel A1.5 m, with *B. subtilis* glutamine synthetase as a standard.

Divalent cation requirement

In previous papers, we reported that the activity of *B. subtilis* and *B. cereus* glutamine synthetase was greatly enhanced by the addition of Mn^{2+} to the Mg^{2+} -dependent assay mixture (13, 17). In *E. coli* glutamine synthetase, the divalent cation specificity is modulated by adenylation/deadenylation of the subunits, and Mg^{2+} -dependent activity is inhibited by the addition of Mn^{2+} (21). The alkalophilic *Bacillus* glutamine synthetase is activated by either Mn^{2+} or Mg^{2+} ; however, the maximum activity was seen when the ratio of Mn^{2+} /ATP was about 1 and the ratio of Mg^{2+} /ATP was more than 2 (Fig. 2A). Cd^{2+} -dependent activity

Fig. 1. SDS-polyacrylamide slab gel electrophoresis of the glutamine synthetase purified from three bacteria on a 12.5% gel.

Lane 1, glutamine synthetase from *Bacillus subtilis* (1 μ g); lane 2, glutamine synthetase purified from alkalophilic *Bacillus* (4 μ g); lane 3, glutamine synthetase from *Escherichia coli* (1.5 μ g).

Fig. 2. Effect of increasing concentration of divalent cations on the velocity of alkalophilic *Bacillus* No. 170 glutamine synthetase activity.

(A) ATP concentration was constant at 5 mM in each assay mixture. (B) At two different ATP/Mg²⁺ ratios (○, ATP/Mg²⁺ was 5 mM/20 mM; ×, ATP/Mg²⁺ was 5 mM/5 mM); and Mn²⁺ was increased to the indicated concentration. Activity measurements were performed by the standard biosynthetic assay at 30°C, as mentioned under 'MATERIALS AND METHODS.'

was similar to that of Mn^{2+} but Co^{2+} -dependent activity was extremely small. In the presence of 20 mM Mg^{2+} , the maximum biosynthetic activity was seen only when 0.2 mM Mn^{2+} was added to the assay mixture (Fig. 2B). The results suggested that the binding sites of Mn^{2+} and Mg^{2+} are separate from each other in the active site(s) of the enzyme. The Cd^{2+} -dependent activity of the glutamine synthetase had unusual properties. The unique effects of the $\text{Mg}^{2+}/\text{Mn}^{2+}$ ratio on the catalytic activity were characteristic of *Bacillus* species glutamine synthetases.

Inhibition by FSBA and iodoacetoamide

To characterize the binding sites of Mg^{2+} and Mn^{2+} , purified enzyme was modified by the ATP analog 5'-*p*-fluorosulfonyl benzoyl adenosine (FSBA), and the effects on Mg^{2+} -, Mn^{2+} -, and Mg^{2+} plus Mn^{2+} -dependent activities were investigated. Figure 3A shows the changes in catalytic activity of alkalophilic *Bacillus* glutamine synthetase by FSBA. Mn^{2+} -dependent activity was decreased to 45% of its initial activity, in parallel to the loss of Mn^{2+} plus Mg^{2+} -dependent activity in 60 min, although Mg^{2+} -dependent activity remained at 80% of its initial activity in the same period. These results suggest that the binding of FSBA to glutamine synthetase affected the Mn^{2+} - and Mn^{2+} plus Mg^{2+} -dependent reactions

Fig. 3. Time course of inactivation by chemical modification of alkalophilic *Bacillus* No. 170 glutamine synthetase.

(A) Glutamine synthetase (0.21 mg/ml) was incubated with 26 μM FSBA at 25°C in 50 mM Tris-HCl buffer (pH 7.2). Each Mn^{2+} - (●), Mg^{2+} - (x), and Mg^{2+} + Mn^{2+} -dependent activity (○) was measured. (B) Glutamine synthetase (0.21 mg/ml) was incubated with 5 mM iodoacetoamide at 25°C in 50 mM Tris-HCl buffer (pH 9.0). Each enzyme activity was assayed as in (A), by the method described under 'MATERIALS AND METHODS.' At time zero, Mn^{2+} -, Mg^{2+} -, and Mg^{2+} + Mn^{2+} -dependent activity were 6.5, 7.4, and 14.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively.

Table 1. Effects of substrates on the inactivation of glutamine synthetase by FSBA.

Addition	Residual activity (%)
None	51
ATP (5 mM)	97
L-Glutamate (50 mM)	22
ATP (5 mM) + L-Glutamate (50 mM)	99

The enzyme was incubated with 0.1 mM FSBA at 25°C for 60 min. The catalytic activity was measured in Mg^{2+} + Mn^{2+} -dependent activity. The results are expressed as a percentage relative to those of assays performed in the absence of FSBA.

more than the Mg^{2+} -dependent reaction. As shown in Table 1, the inactivation by FSBA was completely protected by the addition of 5 mM ATP, and was enhanced by the addition of 50 mM glutamate to the modification mixture. The effect of L-glutamate is significant because the binding of L-glutamate and ATP (E·S complex formation) involves strong synergism. These results suggest that the amino acid residue which reacts with FSBA is at or near the ATP binding site of the enzyme similar to other *Bacillus* enzymes. In each glutamine synthetase from *E. coli* or *B. cereus*, the modified amino acid residues are Lys 47 (19) or Lys 43 (15), respectively.

Modification of a cysteine residue with iodoacetoamide resulted in altered divalent cation dependency of the glutamine synthetases from *B. cereus* (17) and *B. subtilis* (2). In the modified enzyme, both the Mg^{2+} -dependent and the Mg^{2+} plus Mn^{2+} -dependent activities were decreased, but the Mn^{2+} -dependent activity increased. These changes in catalytic activity were qualitatively similar to changes seen upon adenylation of *E. coli* glutamine synthetase (21). Regulation by sulfhydryl group modification in vivo is difficult to establish, however *Bacillus* glutamine synthetases had a unique, reactive cysteine residue. The modified cysteine residue of *B. cereus* was Cys 306 (15) that is conserved in *Bacillus* glutamine synthetase (16,18,22). As shown in Fig. 3B, Mg^{2+} - or Mg^{2+} plus Mn^{2+} -dependent activity of the alkalophilic *Bacillus* enzyme decreased to 50% of the initial activity in 60 min, similar to the activity of *B. cereus* glutamine synthetase (17), and the Mn^{2+} -dependent activity increased to 180%. This suggests that the binding sites of Mn^{2+} and Mg^{2+} are separate from each other in the active site of *Bacillus* glutamine synthetases and the cysteinyl residue which reacted with iodoacetoamide was also very close to the metal·ATP substrate-binding sites on the alkalophilic *Bacillus* glutamine synthetase.

Kinetic properties

Figure 4A shows the pH curves for the biosynthetic reactions using the purified enzyme with either Mn^{2+} , Mg^{2+} , or Mg^{2+} plus Mn^{2+} as divalent cations. The optimum pH varied considerably with the divalent cations used in the assay, however each profile was similar to that of other *Bacillus* enzymes. At pH 9.0, Mg^{2+} plus Mn^{2+} -dependent activity of alkalophilic *Bacillus* enzyme was higher than

Fig. 4. Kinetic properties of alkalophilic *Bacillus* No. 170 glutamine synthetase.

The activity-pH profiles (A) and the glutamate saturation curves (B) were determined with Mg^{2+} (x), Mn^{2+} (●), or $Mg^{2+} + Mn^{2+}$ (○) as a divalent cation under the standard assay condition. Tris-maleate buffer (final concentration of 50 mM Tris and 50 mM maleic acid) adjusted to various pHs with NaOH; the pH of the reaction mixture was measured.

that of *B. cereus* enzyme (13), but the optimum pH of both enzymes was near 7.8. These results suggest that the optimum pH of the enzymes from alkalophilic *Bacillus* does not always depend on their growth condition. Figure 4B shows the substrate saturation curves for glutamate with either Mg^{2+} -ATP, Mn^{2+} -ATP, or Mg^{2+} plus Mn^{2+} -ATP, each of fixed concentration. The apparent K_m for each of the three substrates and V_{max} are shown in Table 2. In the Mg^{2+} plus Mn^{2+} -dependent reaction, the K_m value for each of these three substrates was intermediate between those of the Mn^{2+} - and Mg^{2+} -dependent reactions, and the V_{max} was higher than that for either Mg^{2+} or Mn^{2+} . As shown in Fig. 2B, the activity of the enzyme was greatly enhanced by the addition of Mn^{2+} to the Mg^{2+} -dependent assay mixture, and the highest V_{max} was obtained in the Mn^{2+} plus Mg^{2+} -dependent assay (Table 2). This finding suggests that *Bacillus* species glutamine synthetases are rigorously controlled by the intracellular concentration of divalent cations, in common with glutamine, AMP and other metabolites. From a physiological point of view, the Mg^{2+} plus Mn^{2+} -dependent activity was most important for this *Bacillus* (4).

Effect of feedback inhibitors

In contrast to the *E. coli* enzyme, *B. subtilis* glutamine synthetase is directly inhibited by glutamine. The intracellular level of glutamine is the most important factor in determining *B. subtilis* glutamine synthetase activity (3). Hence, the

Table 2. Kinetic constants of glutamine synthetase from alkalophilic *Bacillus* No. 170.

	Biosynthetic activity		
	Mg ²⁺ -assay	Mn ²⁺ -assay	Mg ²⁺ + Mn ²⁺ -assay ^a
<i>K_m</i> (mM)			
ATP	1.76	0.260	1.03
Glutamate	29.6	1.31	10.4
NH ₃	0.280	1.10	0.50
<i>V_{max}</i> (μmol/min/mg)	11.1	6.88	16.5

^a Mn²⁺ : Mg²⁺ = 1 : 100.

The apparent *K_m* values were obtained from double reciprocal plots of velocity versus substrate concentration.

Table 3. Effect of feedback inhibitors upon biosynthetic activity of glutamine synthetase.

Inhibitor (mM)	Remaining activity (%)		
	Mg ²⁺	Mn ²⁺	Mg ²⁺ + Mn ²⁺
None	100	100	100
Alanine (5)	100	49	87
Glycine (5)	94	55	91
Serine (5)	89	71	99
Glutamine (1)	46	93	76
AMP (0.2)	32	85	54

The enzyme activity was assayed by standard methods described under 'MATERIALS AND METHODS.' One hundred percent activity of Mg²⁺-, Mn²⁺-, and Mg²⁺ + Mn²⁺-dependent reactions were 6.2, 7.5 and 12.9 μmol/min/mg protein, respectively. The enzyme was not preincubated with the inhibitors.

effects of various end-products of glutamine synthetase were examined under the three sets of divalent cation assays with all substrates in a saturated condition. The results of five compounds which appreciably inhibited the activities of the alkalophilic *Bacillus* glutamine synthetase are summarized in Table 3. Glutamine and AMP were the most potent inhibitors at a physiologically low concentration for the Mg²⁺-dependent activity of the enzyme, but the enzyme's sensitivity to the feedback inhibitors was altered by the divalent cation present during the assay. When Mg²⁺ plus Mn²⁺ was used as the divalent cation, the enzyme activity became less sensitive to each inhibitor. These catalytic properties of alkalophilic *Bacillus* glutamine synthetase are compatible with those of *B. cereus* (12) and *B. subtilis* (13) enzyme.

Determination of NH₂-terminal amino acid sequence

The NH₂-terminal amino acid sequences of the glutamine synthetase purified from alkalophilic *Bacillus* was determined by automated Edman degradation with an Applied Biosystems sequencer system in 20 steps. The fifth amino acid remained undetermined, since small quantities of PTP-amino acids corresponding to Arg and Lys were found. A comparison of the NH₂-terminal amino acid sequence of glutamine synthetase from alkalophilic *Bacillus* and eleven other prokaryotes is

	1	10	20
1. Alkalophilic <i>Bacillus</i>	AKYT	XEDIVKLVKEENVKYI	
2. <i>B. subtilis</i> PCI219(18)	AKYT	TREDIVKLVKEENVKYI	
3. <i>B. subtilis</i> (22)	AKYT	REDIEKLVKEENVKYI	
4. <i>B. cereus</i> (16)	ARYT	KEDIFRLAKEENVKYI	
5. <i>Clostridium acetobutylicum</i> (7)	AKYT	KEDIINLVKENGVKFI	
<hr/>			
6. <i>Streptomyces coelicolor</i> S(25)	• FQN	ADDVKKFIADEDDVKFV	
7. <i>Mycobacterium smegmatis</i> (10)	AEKT	SDDIYKLIKDENVEYV	
8. <i>M. phlei</i> (10)	AEKT	ADDILKLIRDDEVEYV	
9. <i>Anabaena</i> 7210 (23)	• • T	TRQEV LKRIQDEKIELI	
10. <i>Thiobacillus ferrooxidans</i> (20)	• GYS	PSDVVKLIQEKDIKFI	
11. <i>Salmonella typhimurium</i> (6)	• • •	SAEHLVTMLNEHEVKFV	
12. <i>Escherichia coli</i> (1)	• • •	SAEHLVTMLNEHEVKFV	

Fig. 5. NH₂-terminal amino acid sequence.

The line may be conveniently divided into two groups: those above the line are spore-forming bacteria, and those below are subject to regulation by adenylation except *Anabaena*.

shown in Fig. 5. Although the analysis was limited, the sequence of alkalophilic *Bacillus* glutamine synthetase was in agreement with the sequence from two strains of *B. subtilis* and *B. cereus* glutamine synthetase (16, 18, 22), and demonstrated high homology with the enzyme from *Clostridium acetobutylicum* (7). The sequence of *Bacillus* glutamine synthetase is not similar to those from the three other Gram-positive bacteria, such as *Streptomyces* (25) and *Mycobacterium* (10), nor to those from Gram-negative bacteria. Although the activity of *Anabena* glutamine synthetase can not be regulated, the other six glutamine synthetase activities can be regulated by adenylation/deadenylation similar to the *E. coli* glutamine synthetase. The results of an Ouchterlony double immunodiffusion experiment of alkalophilic *Bacillus* glutamine synthetase versus antisera of *B. subtilis* glutamine synthetase showed high reactivity similar to that of each glutamine synthetase from *B. subtilis* or *B. cereus* (13).

In conclusion, it is clear that glutamine synthetases from *Bacillus* species do not appear to have a global regulation system of nitrogen metabolism as demonstrated in *E. coli* (5, 11, 14, 21, 24), though they can sporulate when deprived of a rapidly metabolizable nitrogen source. Apparently, many properties of the alkalophilic *Bacillus* enzyme are dependent on the divalent cation used for activation, similar to the enzymes from *B. subtilis* and *B. cereus*. Thus, the Mg²⁺/Mn²⁺ ratios may be important in the regulation of the *Bacillus* glutamine synthetase under physiological conditions.

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