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Purification and Properties of Glutamine Synthetase from *Hydrogenobacter thermophilus* TK-6

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Abstract

Hydrogenobacter thermophilus TK-6, a thermophilic and obligately chemoautotrophic bacterium, assimilates ammonium using glutamine synthetase (GS). GS was purified using three chromatography steps. The purified GS was found to belong to GS type I on the basis of its subunit composition and molecular weight. The Mg²⁺-dependent activity of this GS significantly increased after incubation with phosphodiesterase, indicating that GS is subject to adenylyl/deadenylyl regulation, a posttranslational modification system reported mainly among enterobacteria. The degree of this posttranslational modification changed depending on growth phase, confirming that adenylyl/deadenylyl regulation functions *in vivo*. Interestingly, the *K_m* for glutamate of *H. thermophilus* GS was significantly higher than those of other organisms, suggesting that GS activity is affected by intracellular glutamate concentration.

Key words

Glutamine synthetase, *Hydrogenobacter thermophilus*, Nitrogen assimilation, Anabolism, Adenylylation

Introduction

Glutamine synthetase (GS) is one of the most important enzymes in nitrogen metabolism (1). It produces glutamine from glutamate and ammonia, accompanying ATP hydrolysis. Although GS is essential for glutamine synthesis, it also plays a crucial role in supplying nitrogen for other nitrogen-containing metabolites. GS couples with glutamate synthase (GOGAT), which produces 2 mol of glutamate from 1 mol of glutamine and 1 mol of 2-oxoglutarate, and the resulting glutamate molecules act as ni-

trogen donors to various metabolites. This coupling reaction is known as the GS/GOGAT pathway and is a major ammonium assimilatory pathway in many organisms (1, 2).

GS is classified into three types (1): GS type I (GSI) is widely distributed among many organisms, including *Bacteria*, *Archaea*, and *Eukarya*, and is considered to be one of the oldest existing enzymes (3). GSI is a homododecamer composed of subunits with a molecular mass of 55 kDa. GS type II was identified in plant-symbiotic bacteria and eukaryotes as a homooctamer composed of subunits with a molecular mass of 36 kDa. GS type III was discovered in cyanobacteria to be a homohexamer composed of subunits with a molecular mass of 75 kDa (4, 5). In contrast to GSI, GS types II and III have been found in a limited number of organisms.

GS plays an important role in nitrogen metabolism as mentioned above, but excess GS activity causes the depletion of the intracellular carbon skeleton. Owing to its effect on both nitrogen and carbon metabolisms, GS activity is strictly regulated *in vivo* by several regulatory systems, such as those involving product inhibition (6) and protein-protein interaction (7). One of the best known GS regulatory mechanisms is the adenylyl/deadenylyl system (1). In this system, GS is activated and inactivated by adenylyltransferase through deadenylylation and adenylylation, respectively. Furthermore, adenylyltransferase is found in a regulatory cascade that responds to nutrient conditions, such as nitrogen and carbon availability. While this adenylyl/deadenylyl regulatory system is distributed in GSIs mainly among gram-negative enterobacteria, there are GSIs that are not subject to this regulation in cyanobacteria and gram-positive bacteria (7, 8).

Hydrogenobacter thermophilus TK-6 is an obligately chemoautotrophic, hydrogen-oxidizing, thermophilic bacterium. It uses hydrogen as the sole energy source using hydrogenases (9) and carbon dioxide as the sole carbon source via the reductive tricarboxylic acid cycle (RTCA cycle) (10). *H. thermophilus* has several unique characteristics in its pathway and enzymes for carbon metabolism (11-15). However, its pathway and enzymes for nitrogen metabolism have yet to be determined. Its physiological characteristics are also intriguing from the view of the phylogeny of the organism, because *Hydrogenobacter* species are located with *Aquifex* species on the deepest branch in the *Bacterial* domain determined on the basis of 16S ribosomal RNA sequence analysis (16, 17).

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In this study, we detected GS activity in *H. thermophilus*. To clarify the features of the key enzyme in nitrogen assimilation, we purified GS from *H. thermophilus* and determined its enzymatic characteristics. Our results show that GS activity in *H. thermophilus* is regulated by the adenylyl/deadenylyl system and intracellular glutamate concentration.

Materials and methods

Bacterial strain and growth conditions

H. thermophilus TK-6 (IAM 12695) was cultivated as previously described (18). The culture medium contained 3 g/l $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source.

GS assay

GS activity was assayed by measuring the production of inorganic phosphate (19) at 70 °C, the growth temperature for *H. thermophilus*. The reaction mixture contained 100 mM HEPES-KOH (pH 6.8 at 70 °C), 15 mM ATP, 200 mM glutamate, 20 mM NH_4Cl , 50 mM MgSO_4 and an enzyme solution in a total volume of 100 μl . The reaction mixture was preincubated for 2 min, and the reaction was started by adding 5 μl of the enzyme solution. After 6 min of incubation at 70 °C, the reaction was stopped by chilling the tube with ice-cold water, and 900 μl of ferrous sulfate solution (0.008 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 ml of 7.5 M H_2SO_4) and 75 μl of ammonium molybdate solution (0.066 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 1 ml of 3.75 M H_2SO_4) were added to the reaction mixture. Inorganic phosphate concentration was determined by measuring absorbance at 660 nm. A calibration curve was plotted using a KH_2PO_4 solution. One unit of activity was defined as the activity producing 1 μmol of inorganic phosphate per minute. In Mn^{2+} -dependent assays, 50 mM MgSO_4 was replaced with 2 mM MnSO_4 .

GS purification

H. thermophilus TK-6 cells were harvested by centrifugation at 5000 $\times g$ for 10 min when the optical density at 540 nm reached about 2.5. The cells (10 g of wet cells) were washed with 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. Cell debris was removed by centrifugation at 100,000 $\times g$ for 1 h. The supernatant (designated as cell-free extract, CFE) was applied to a DE52 open column (Φ 25 mm $\times$ 15 cm; Whatman, Brentford, Middlesex, UK) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . After eluting of bound proteins with a buffer containing 1 M NaCl, ammonium sulfate was added to the obtained fraction to 30% saturation, and the sample was applied to a Butyl-Toyopearl column (Φ 22 mm $\times$ 15 cm; Tosoh, Tokyo) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and ammonium sulfate at 30% saturation. This and subsequent chromatography steps were performed using an ÄKTA purifier system (GE Healthcare, Piscataway, NJ,

USA). Proteins were eluted with a linear gradient of ammonium sulfate from 30% to 0% with a total gradient volume of three column volumes at a flow rate of 4 ml $\cdot$ min⁻¹. GS was eluted with ammonium sulfate at approximately 13% saturation. The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 , and were applied to a DEAE-Toyopearl column (Φ 22 mm $\times$ 15 cm; Tosoh) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . Proteins were eluted with a linear gradient of NaCl from 0 M to 1 M with a total gradient volume of five column volumes at a flow rate of 4 ml $\cdot$ min⁻¹. GS eluted at approximately 0.34 M NaCl. The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 , and were applied to a MonoQ HR 5/5 column (bed volume 1 ml; GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . Proteins were eluted with a linear gradient of NaCl from 0 M to 1 M with a total gradient volume of 40 column volumes at a flow rate of 0.5 ml $\cdot$ min⁻¹. GS eluted at approximately 0.32 M NaCl. The active fractions were designated as purified GS, and stored at -80 °C until use.

Protein assay

Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). A calibration curve was plotted using bovine serum albumin as a standard protein.

Gel filtration

To estimate the molecular mass of GS, gel filtration was performed using a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer containing 1 mM MgCl_2 and 150 mM NaCl, at a flow rate of 0.5 ml $\cdot$ min⁻¹. Chromatography was performed using an ÄKTA purifier system (GE Healthcare). Gel filtration standard (Bio-Rad, Hercules, CA, USA) and ferritin (Boehringer, Ingelheim, Germany) were used as molecular makers for the calibration. Each measurement of standards and samples was performed in triplicate.

GS Deadenylylation

For GS deadenylylation, phosphodiesterase I from snake venom (Sigma, St. Louis, MO, USA) was used (20). Reaction mixtures containing 20 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 2 mg/ml phosphodiesterase, and an enzyme solution with a GS activity of 0.11 U/ml were incubated at 37 °C for 4 h. Owing to the composition of this mixture, the Mn^{2+} -dependent GS assay mixture was contaminated with a low concentration of Mg^{2+} (up to approximately 250 μM). Reaction mixtures that contained all the components except phosphodiesterase were prepared as negative controls.

Kinetic characterization

For determining the optimum pH of GS, GS activity

was measured in HEPES-KOH buffer at pHs 5.8, 6.3, 6.8, 7.3 and 7.8 at 70 °C. Purified GS that was not deadenylylated by phosphodiesterase *in vitro* was used for the reactions.

K_m was determined by varying the concentration of glutamate from 3.3 to 200 mM, that of ATP from 0.2 to 15 mM, and that of NH_4Cl from 0.1 to 50 mM. Purified GS was deadenylylated by phosphodiesterase *in vitro* before the K_m assays.

TABLE 1. Purification of GS from *Hydrogenobacter thermophilus* TK-6

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (-fold)	Yield (%)
CFE	29.4	403	0.073	1	100
Butyl	28.8	32	0.90	12	98
DEAE	15.1	1.7	8.9	122	51
MonoQ	12.4	1.1	11	154	42

FIG. 1. SDS-PAGE (10%) of purified GS. Lane 1, Molecular mass markers; lane 2, purified GS.

Results

Purification of GS from *H. thermophilus*

GS activity was detected in the CFE of *H. thermophilus*. Specific GS activity was estimated to be higher than $0.4 \text{ U} \cdot (\text{mg protein})^{-1}$ in CFE from mid-log phase cultures. The activity drastically decreased when the cells reached the stationary phase (see below).

GS was purified from late-log phase *H. thermophilus* cells, and 1.1 mg of purified enzyme was obtained from 10 g of wet cell paste (Table 1). The purified GS showed a single band at 55 kDa on SDS-PAGE (Fig. 1), similarly to known GSIs from other organisms.

To determine the subunit composition, gel filtration was performed with the purified GS. Because the enzyme eluted slightly faster (12.52 ml of elution volume) than the largest molecular standard (thyroglobulin, 670 kDa; 12.75 ml of elution volume), its molecular mass could not be accurately calculated. However, it is highly possible that the enzyme is a dodecamer, similarly to GSIs from other organisms (19).

GS Deadenylation

GSIs from some organisms are subject to adenylyl/deadenylation regulation. Therefore, we determined whether purified GS could be deadenylylated by phosphodiesterase from snake venom.

After incubating purified GS with phosphodiesterase, Mg^{2+} -dependent activity showed a 6-fold increase, whereas Mn^{2+} -dependent activity decreased slightly (Table 2). Because deadenylylated and adenylylated GSs have high Mg^{2+} -dependent and low Mn^{2+} -dependent activity, respectively (19), changes in the Mg^{2+} -dependent and Mn^{2+} -dependent activities after phosphodiesterase treatment suggest that the purified GS from *H. thermophilus* had been partially inactivated by adenylylation. These results also suggest that the GS from *H. thermophilus* belongs to GSI.

To determine whether GS activity is also regulated by

FIG. 2. Growth curve of *H. thermophilus* (A) and GS specific activities in CFE after incubation with and without phosphodiesterase (B). (A) Arrows indicate sampling points for GS assays. (B) GS activities in CFE obtained from cells after 5, 9, 13 and 17 h of cultivation. Each sample was incubated with and without phosphodiesterase. Open and closed bars indicate the specific activities after incubation without and with phosphodiesterase, respectively.

TABLE 2. Mg^{2+} - or Mn^{2+} -dependent GS activity after incubation with or without phosphodiesterase

Cation	Specific activity (U/mg-protein)	
	Without PDE	With PDE
Mg^{2+}	11	66
Mn^{2+}	15	9
None	< 2	< 2

PDE, Phosphodiesterase.

TABLE 3. K_m values for reaction substrates

Species	K_m (mM)			Ref.
	Glu	NH_4^+	ATP	
<i>H. thermophilus</i> ^a	32±2	0.12±0.01	0.67±0.09	
<i>Escherichia coli</i>	2.4	1.8	0.68	21
<i>Pseudomonas aeruginosa</i>	1.6	0.2		22
<i>Azotobacter vinelandii</i>	1.2	0.15	0.94	23
<i>Methylococcus capsulatus</i>	2.25	0.72	0.60	6
<i>Synechocystis</i> PCC 6803	1.2	0.17	0.55	24
<i>Calothrix</i> PCC 7601	0.35	0.065	0.30	24

^a Standard errors are indicated after the ± symbol.

adenylation *in vivo*, we measured GS specific activity in CFEs sampled periodically from various growth phases, namely, from the mid-log phase to the stationary phase (Fig. 2A). Although *H. thermophilus* had high GS activity during the mid-log phase (above $0.4 \text{ U} \cdot [\text{mg protein}]^{-1}$), the activity without phosphodiesterase treatment rapidly decreased along with the growth phase transition (Fig. 2B). In contrast, no such a marked decrease in GS activity was observed after the deadenylylation treatment with phosphodiesterase. These results indicate that the decrease in GS activity was caused not by transcriptional or translational down-regulation, but by posttranslational adenylylating inactivation.

Kinetic characterization

When GS activity was measured in assay mixtures at various pHs, the maximum GS activity was detected at pHs 6.3 - 6.8.

The purified GS exhibited Michaelis-Menten kinetics when the concentration of one of the substrates was varied. The K_m values for glutamate, ammonium, and ATP are shown in Table 3. Interestingly, the GS from *H. thermophilus* has a notably high K_m for glutamate in contrast to GSs from other organisms, although its K_m values for ammonium and ATP are comparable to those of other GSs. These K_m values indicate that the GS from *H. thermophilus* has special characteristics with respect to the reactivity with glutamate.

Discussion

In this study, we purified GS from *H. thermophilus*, and determined its enzymatic characteristics and adenylylating regulation system. The K_m for glutamate was extraordinarily high, suggesting that GS activity in *H. thermophilus* is affected by intracellular glutamate concentration. A similar kinetic property was reported in the glucokinase of the rat liver (25). The enzyme has a high K_m for glucose, a property considered to enable the control of enzymatic activity in response to a change in glucose concentration.

The sensitivity of GS to glutamate concentration may be related to the carbon metabolism of *H. thermophilus*. This organism assimilates carbon dioxide through the RTCA cycle. Because 2-oxoglutarate, a precursor of glutamate, is an intermediate in this central carbon anabolism, intracellular glutamate concentration may be more dependent on carbon availability in *H. thermophilus* than in other organisms that uptake carbon through pathways other than the RTCA cycle. Probably, the high K_m for glutamate enables the carbon metabolism to modulate the nitrogen assimilation flux by adjusting GS activity.

Posttranslational modification by adenylylation has been observed in GSIs from such bacteria as *Escherichia coli* (19), *Pseudomonas aeruginosa* (22), and *Streptomyces cattleya* (26). However, GSIs from *Bacillus subtilis* (8), *Clostridium acetobutylicum* (27), cyanobacteria (7), and archaea (28) are not subject to this regulation. It is not clear when the adenylyl/deadenylyl mechanism evolved, even though the gene encoding GSI has been suggested to be one of the oldest existing genes on the basis of phylogenetic evidence (3). In this study, an adenylyl/deadenylyl mechanism was found in *H. thermophilus*, which is located on the deepest branch of *Bacteria* in the phylogenetic tree of 16S ribosomal RNA sequences. This fact suggests that the adenylylating regulation of GSI originated before the divergence of the genus *Hydrogenobacter* from other bacteria. This regulatory system has been conserved in many organisms, however, some organisms, such as *B. subtilis*, are supposed to have developed other regulatory systems and to have lost the adenylylating system.

In many organisms, GS couples with GOGAT for ammonium anabolism, and GOGAT activity has been also detected in *H. thermophilus*. Our study of the purification and characterization of GOGAT from *H. thermophilus* will be published elsewhere.

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