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Purification and Characterization of Glutamine Synthetase from the Archaebacterium *Methanobacterium ivanovi*

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Glutamine synthetase (GS) was purified to electrophoretic homogeneity from the obligate anaerobic archaebacterium *Methanobacterium ivanovi*. The 130-fold-purified enzyme was obtained by heat treatment, ion-exchange chromatography, and gel filtration. Like all other eubacterial GSs known so far, the GS of *M. ivanovi* was found to be a dodecamer of about 600,000 daltons composed of a single type of subunit. The enzyme was stable at 63°C for 10 min and was not sensitive to oxygen. The isoelectric point was 4.6, and the optimum pH of gamma-glutamyltransferase activity was 8.0. The K_m values for hydroxylamine, glutamine, and ADP in the transferase reaction were 6.8, 22.7, and 0.35 mM, respectively. L-Methionine-DL-sulfoximine strongly inhibited the activity. Like the GS from gram-positive bacteria, *Anabaena* sp., several yeasts, and mammals, the enzyme from *M. ivanovi* was not regulated by adenylylation as demonstrated by snake venom phosphodiesterase treatment. Inhibition of the transferase activity by L-alanine, glycine, L-histidine, and L-tryptophan was observed. L-Glutamine alone or in the presence of AMP did not inhibit the GS synthetic activity. The GS of *Methanobacterium ivanovi* did not cross-react with a variety of antisera against GS from *Escherichia coli*, *Anabaena* strain 7120, or *Bacillus megaterium*. Archaebacterial GS appears to be structurally and functionally similar to eubacterial GS in gram-positive bacteria.

Glutamine synthetase (GS) (L-glutamate:ammonia ligase (ADP); EC 6.3.1.2) plays a key role in the ammonia assimilation in many procaryotes and eucaryotes since it catalyzes the first step in a metabolic pathway that leads finally to the synthesis of nearly all the important macromolecules of the cell (23, 28). It has been extensively studied at the biochemical and molecular level in various bacteria (2, 28, 36) and cyanobacteria (25, 29, 34, 36). Many reports are also available on the GSs of fungi, plants, and mammals (28). In contrast, little information is available on the enzymes involved in the nitrogen metabolism and ammonia assimilation by methanogens placed in the third kingdom of life, the archaebacteria (4, 19).

Methanogens are strict anaerobes capable of producing methane from H_2-CO_2, formate, methanol, acetate, etc. (15, 41). Methanogens, without exception, can utilize ammonia as a nitrogen source for growth (41), although recently, it has been shown that *Methanobacterium ivanovi* can also utilize glutamine and that *Methanobacterium thermoautotrophicum* delta H is capable of growth on urea or glutamine (10) as a nitrogen nutrient source. Bryant et al. (12) studied the nitrogen requirements of *Methanobacterium* strain MOH and *Methanobacterium ruminantium*. These organisms required ammonia as a nitrogen source with relatively little nitrogen being incorporated from amino acids supplied. An important contribution to the study of nitrogen metabolism in methanogens has recently been reported in which pure cultures of *Methanococcus thermolithotrophicus* (5), *Methanosarcina barkeri* 227 (25) and *Methanosarcina barkeri* (11) have been shown to fix nitrogen. A genetic support to this observation has been provided in our laboratory by demonstration that DNA from *Methanobacterium ivanovi* and a few other methanogens hybridizes with *Klebsiella pneumoniae* and *Anabaena* strain 7120 *nifKDH* gene probes (32).

Recently, the mechanism of ammonia assimilation was studied in *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum* (20). Both methanogens contained significant activities of GS, glutamate synthase, and several transaminases. Alanine dehydrogenase was also detected in *Methanobacterium thermoautotrophicum*. Reports on the purification and characterization of any of the enzymes involved in ammonia assimilation by methanogens are not yet available. Thus, the present study was undertaken to purify the GS from *Methanobacterium ivanovi* and to characterize its structural and biochemical properties, regulation, role in ammonia assimilation, and relationship with other eubacterial and eucaryotic enzymes.

MATERIALS AND METHODS

Chemicals, gases, and purification materials. All chemicals, gases, and reagents used were of analytical grade. Gases supplied by Carboxyque Francaise or L'Air Liquide (Paris, France) were passed over heated copper fillings or catalyst BASF Ro-20 at room temperature to remove traces of oxygen. DEAE-Sephacel, Sephacryl S-300, columns, polybuffer 74, and PBE94 gel for chromatofocusing and molecular weight standards for gel filtration were obtained from Pharmacia France S.A. (Bois d'Arcy, France). Ultrogel AcA22 and the fraction collector (LKB 2111 MultiRac) along with accessories were from LKB Instruments S.A. (Orsay, France). Indubiose-A37 was purchased from IBF Pharmindustrie (Clichy, France).

Organism and cultivation. *Methanobacterium ivanovi* (6; M. K. Jain, T. Thompson, and J. G. Zeikus, submitted for publication) was routinely grown at 37°C with agitation under an H_2-CO_2 (80:20) atmosphere in phosphate-buffered basal minimal medium with 20 mM NH_4Cl as described by Bhatnagar et al. (8, 9). It was mass cultured for GS purifi-

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cation in a 20-liter fermentor (Chemapac SARL, Bron, France) containing 15 liters of phosphate-buffered basal minimal medium. The pH was regulated between 7.2 and 7.4 by the addition of 6 N NaOH. A mixture of H_2-CO_2 (80:20) was continuously flushed at 300 ml/min through the medium which was agitated at 500 rpm. To maintain the required sulfide concentration, 20 ml of a 2.5% $Na_2S \cdot 9H_2O$ solution was added every day from day 2 onward. The temperature was regulated at 37°C. After inoculation, the initial optical density was $A_{660} = 0.035$ and the final optical density was $A_{660} = 2.3$. Cells were harvested after 4 days by centrifugation under argon in a cooled Sharples centrifuge. The cell paste was frozen and stored in liquid nitrogen until used. Growth was monitored by methane production and A_{660} measurements and was examined by microscopy during the fermentation time course.

Preparation of extracts and purification steps. (i) Crude extract preparation. All operations were carried out at 4°C under aerobic conditions unless otherwise specified. Frozen cell paste (5 g) was suspended in 10 ml of buffer containing 10 mM imidazole hydrochloride (pH 7.0), 50 mM $MgCl_2$, and 1 mM mercaptoethanol (buffer A). After washing, bacteria were disrupted in a French pressure cell at 18,000 lb/in^2. The broken-cell suspension was centrifuged at 48,000 × *g* for 30 min. The supernatant was designated as crude extract.

(ii) Streptomycin precipitation. Streptomycin sulfate (0.5 g/ml) was added drop by drop with slow mixing to the crude extract (pH preadjusted to 5.8 with IM acetic acid) to a final concentration of 1 mg/mg of protein. After 15 min at 4°C, the mixture was centrifuged at 48,000 × *g* for 30 min.

(iii) Heat treatment. The supernatant after the streptomycin precipitation step was heated at 63°C for 10 min with slow mixing. The preparation was allowed to cool (4°C) and centrifuged at 48,000 × *g* for 30 min. The supernatant was dialyzed against 200 volumes of buffer A (with three changes).

(iv) DEAE-Sephacel chromatography. The dialyzed solution, diluted 1:1 (vol/vol) with distilled water, was loaded onto a DEAE-Sephacel column (8.0 by 2.5 cm) previously equilibrated with buffer A. The fractions eluted with 75 mM KCl that displayed a GS specific activity greater than 1.5 U/mg of protein were pooled and concentrated 10-fold by ultrafiltration through an Amicon YM 10 Diaflo membrane.

(v) Gel filtration. The concentrated pooled fractions were loaded onto an Ultrogel AcA22 (LKB) column (70.0 by 1.5 cm) previously equilibrated with buffer A, and the enzyme was eluted with the same buffer at a flow rate of 4 ml/h.

GS assay. GS was assayed for both transferase and synthetic activities. The gamma-glutamyltransferase assay for GS activity was done as described by Bender et al. (7). The assay mixture was adapted from that of Shapiro and Stadtman (31) except that disodium hydrogen arsenate was used instead of potassium arsenate. The assay mixture (18 mM hydroxylamine hydrochloride, 0.27 mM $MnCl_2$, 25 mM disodium hydrogen arsenate, and 0.36 mM ADP in 135 mM imidazole hydrochloride buffer) was prepared daily. For controls, ADP and arsenate solutions were replaced by water. After the pH of the assay mixture and blank was adjusted to 8.0 with 2 M KOH, the solutions were kept at 4°C until use during the day. To 0.4 ml of the assay mixture, sample and water were added to give a final volume of 0.45 ml, which was equilibrated for 5 min at 37°C in a water bath. The reaction was initiated by adding 0.05 ml of 0.2 M L-glutamine (20 mM final concentration). Stop mixture (1 ml) (55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, and 21 ml of concentrated HCl per liter) was added to terminate the reaction. The mixture was vortexed and centrifuged to remove any precipitate before reading the A_{540}. Under these conditions, 1 μmol of glutamyl hydroxamate gives 0.533 units of A_{540}. One unit of GS activity was defined as the amount of enzyme producing 1 μmol of glutamyl hydroxamate per min.

The synthetic assay was performed with purified enzyme preparations and measured the ability of GS to form glutamine, as described previously (7). The enzyme preparation (50 μl) was added to 0.4 ml of assay mixture (46 mM hydroxylamine hydrochloride, 55 mM $MgCl_2$, 164 mM monosodium L-glutamate, 92 mM imidazole hydrochloride buffer, pH 7.0). After 5 min at 37°C, the reaction was initiated with 60 μl of 0.2 M ATP (pH 7.0). Controls without ATP were run in parallel. The reaction was terminated by the addition of 1 ml of stop mixture, and the absorbancy was measured at 540 nm.

SVP treatment. Snake venom phosphodiesterase (SVP; phosphodiesterase I (venom); Worthington Diagnostics, Freehold, N.J.) was used. SVP treatment was done as described previously (37). The partially purified GS-containing fraction, after DEAE-Sephacel column elution, was treated with 10 μg of SVP in 100 mM Tris hydrochloride buffer (pH 9.0) containing 1 mM $MgCl_2$ for 3 h at 37°C. Treated enzyme preparation was then assayed for GS activity by the transferase and synthetic reactions as described above in the presence of Mn^{2+} (0.27 mM $MnCl_2$) or Mg^{2+} (55 mM $MgCl_2$) or Mn^{2+} plus Mg^{2+}.

Protein concentration. Protein estimation was performed by the method of Sedmak and Grossberg (30), using Coomassie brilliant blue G-250 with bovine serum albumin as a standard.

Electrophoresis and molecular weight determination. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (22). Protein standards (Bio-Rad Laboratories, Richmond, Calif.) employed were: phosphorylase *b* (92,500 molecular weight), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). Silver staining of the slab gels was done by the method of Ansorge (3). Disc electrophoresis of the native enzyme was performed by the method of Davis (16) in gels (6.5 by 0.8 cm) containing 5% polyacrylamide. For assaying GS activity, 3-mm gel slices were preincubated for 30 min at 37°C with twofold-concentrated assay mixture, and the reaction was initiated with 40 mM glutamine. Native enzyme molecular weight was determined by gel filtration on a Sephacryl S-300 column (50 by 0.9 cm) equilibrated with 50 mM imidazole hydrochloride (pH 7.0)–50 mM NaCl–1 mM $MgCl_2$ at a flow rate of 7 ml/h. Protein standards used were: thyroglobulin (669,000 molecular weight), ferritin (440,000), and aldolase (158,000).

Chromatofocusing. Isoelectric focusing on an ion exchanger (33) was performed with a PBE94 gel column (2-ml bed volume) and polybuffer 74 to generate a pH gradient of 7 to 4 and to elute the enzyme as a function of its isoelectric point at a flow rate of 10 ml/h.

Immunodiffusion. Immunological characterization of the GS from *Methanobacterium ivanovi* was done by using the method of Ouchterlony (37). Gels contained 0.85% of Indubiose-A37 in 10 mM imidazole hydrochloride buffer (pH 7.0)–0.1 M KCl–1 mM mercaptoethanol. Crude extracts and pure enzyme preparations containing 0.1 to 1.0 U of GS activity were used against varied amounts of crude antisera (0, 10, 20, 30, 40, and 50 μl). The antisera were kindly provided by R. Hohman, National Institutes of Health,

TABLE 1. Purification of GS from *Methanobacterium ivanovi*[a]

Step	Vol (ml)	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg of protein)	Yield (%)	Purification factor
1. Crude extract	11.0	132.5	30.26	0.23	100	1
2. Streptomycin precipitation	10.0	94.3	27.35	0.29	90	1.3
3. Heat treatment	9.5	40.8	24.5	0.6	81	2.6
4. DEAE-Sephacel	34.0	12.9	15.5	1.2	51.2	5.2
5. Ultrogel AcA22	15.0	0.13	3.93	30.23	12.9	130

[a] GS activity was determined by transferase reaction. One unit is defined as the amount of enzyme producing 1 μmol of glutamyl hydroxamate per min.

Washington, D.C. (*Escherichia coli* GS antiserum); R. Haselkorn, University of Chicago, Chicago (*Anabaena* strain 7120 GS antiserum); and G. Reysset, Pasteur Institut, Paris (*Bacillus megaterium* GS antiserum).

RESULTS

Purification and analysis of GS activity. Table 1 summarizes the different steps of the purification procedure. The enzyme was purified 130-fold by this procedure. GS in crude extracts was only detected by the gamma-glutamyltransferase reaction in *Methanobacterium ivanovi*. In addition to the transferase reaction, the biosynthetic and synthetic reactions were detectable after the third step of purification. The transferase reaction required the presence of arsenate, ADP, and Mn^{2+}. *Methanobacterium ivanovi* being an obligate anaerobe (6), the GS activity was assayed under aerobic and anaerobic conditions. No significant difference in the specific activity was observed (data not shown). This permitted the assay and the purification procedure to be conducted under aerobic conditions.

Heat treatment at 63°C for 10 min showed that the total GS activity did not decrease; thus, heat treatment was eventually used as a step for purification of GS, as was the case for other organisms. More than 30% of the contaminating proteins were eliminated at this step, and the specific activity increased two to threefold. Ammonium sulfate precipitation was not used because of low enzyme recovery. The property of *E. coli* GS to form highly insoluble paracrystalline aggregates in the presence of Zn^{2+} and Mg^{2+} mixtures (24), was used to try to precipitate the enzyme from *Methanobacterium ivanovi*. A relatively high concentration of Zn^{2+} (e.g., 20 mM) was required to precipitate 60 to 70% of the enzyme activity, whereas in the case of *E. coli* over 95% of the enzyme was precipitated with 1.5 mM Zn^{2+} (24). The *Methanobacterium ivanovi* GS-Zn^{2+} complex (with 20 mM Zn^{2+}) could not be resolubilized, and thus, further purification by gel filtration or ion-exchange chromatography was not possible.

After heat treatment and centrifugation, the supernatant was dialyzed overnight and diluted before loading onto the DEAE-Sephacel column. The subsequent stepwise development of a KCl gradient allowed the elution of the enzyme at the beginning of the 75 mM KCl gradient. The fractions having a specific activity of more than 1.5 U/mg of protein (from the middle of the activity peak) were pooled and loaded onto Ultrogel AcA22 after concentration by ultrafiltration. Pure enzyme thus obtained after gel filtration was observed as single band of about 50,000 molecular weight on a silver-stained polyacrylamide-sodium dodecyl sulfate gel (Fig. 1). The GS activity was localized in the single band obtained in polyacrylamide (5%) native tube-gel by assaying 3-mm cut slices (Fig. 2) by transferase reaction.

Properties of GS. As demonstrated by gel filtration, the molecular weight of the GS was calculated to be about 600,000 (Fig. 3). This indicates that the enzyme is a dodecamer composed of a single type of subunit since the molecular weight of the subunit was observed to be about 50,000 (Fig. 1) on sodium dodecyl sulfate-polyacrylamide gels.

The optimum pH of the gamma-glutamyltransferase reaction assay was 8.0. The synthetic activity showed a broad pH maximum around 7.5. The isoelectric point of the enzyme was 4.6 as determined by chromatofocusing.

The K_m values for the Mn^{2+}-dependent transferase reaction were determined for the substrates hydroxylamine, glutamine, and ADP. A partially purified GS preparation (after heat treatment) was used. Two of the substrates were used at the maximal concentrations, and the concentration of the third reactant was varied. The double-reciprocal plots of velocity versus variable substrate concentration were linear. The apparent K_m values for hydroxylamine, glutamine, and ADP were 6.8, 22.7, and 0.6 mM, respectively. The apparent V_{max} for the three substrates varied between 0.6 and 0.67 U/mg of protein. The specific activity of the pure GS was found to be 17.8 and 30.3 U/mg of protein as measured by the synthetic and transferase reactions, respectively.

Immunodiffusion studies were undertaken to characterize immunologically the GS of *Methanobacterium ivanovi* and

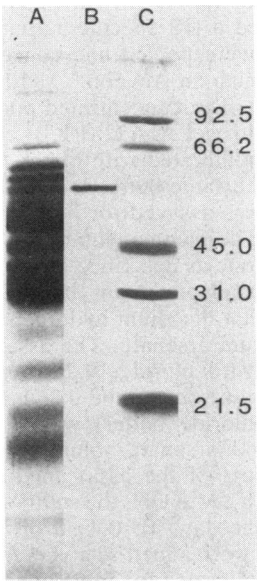

FIG. 1. Silver-stained sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoretic analysis of GS purification from crude extract of *Methanobacterium ivanovi*. Lanes: A, heat-treated extract, 6.1 μg of protein; B, purified GS, 0.3 μg of protein; C, molecular weight ($\times 10^3$) standards (Bio-Rad).

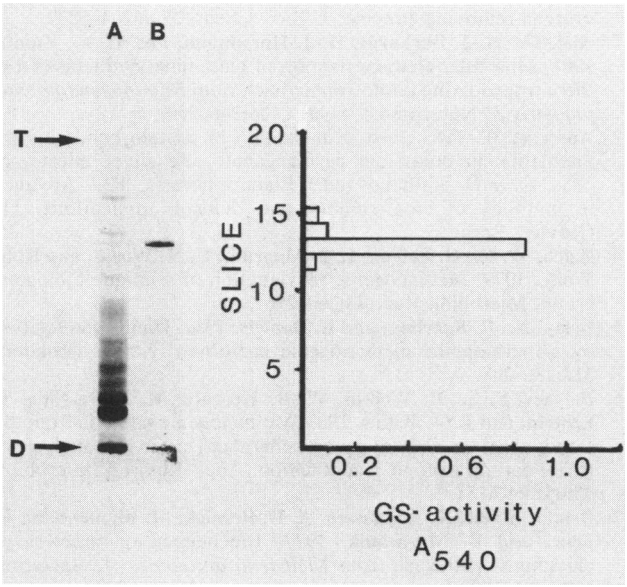

FIG. 2. Native polyacrylamide (5%) gel electrophoresis of purified GS and localization of its activity. Lanes: A, heat-treated extract, 35 μg of protein; B, purified GS, 4 μg of protein. T, Top of resolving gel; D, China-ink needle marking of dye front. Lanes A and B were strained with Coomassie brilliant blue. Activity staining was done with 0.3-cm cut slices of a duplicate, unstained lane B as shown on the right by transferase reaction with twofold-concentrated assay mix. GS activity is expressed as units of A_{540} per slice per hour.

to study its antigenic relationship with the enzymes from other origins. Crude antisera to *E. coli*, *B. megaterium*, and *Anabaena* strain 7120 GSs were individually tested for cross-reactivity with the *Methanobacterium ivanovi* GS by immunodiffusion on Ouchterlony plates. Precipitin bands were not detected in any case.

Regulation of GS activity. It is established that the alteration of GS activity by SVP treatment is presumptive evidence that the enzyme contains adenylylated (or perhaps other nucleotidylated) subunits (35). There was no change in the GS activity of *Methanobacterium ivanovi* after SVP treatment. The GS activity, measured by the transferase and synthetic reactions in the presence of Mn^{2+} or Mg^{2+} or Mn^{2+} plus Mg^{2+}, also remained unchanged after SVP treatment. This was done because it had been reported that the transferase activity of adenylylated *E. coli* GS measured in the presence of 0.3 mM Mn^{2+} is unchanged after SVP treatment, whereas activity in the presence of both 0.3 mM Mn^{2+} and 60 mM Mg^{2+} is greatly increased (35). These results suggest that the GS of *Methanobacterium ivanovi* lacks covalently bound nucleotide and hence is apparently not regulated by the adenylylation-deadenylylation system.

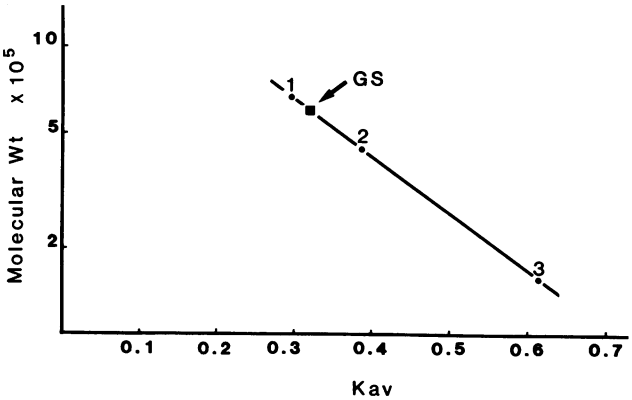

FIG. 3. Molecular weight determination of native GS from *Methanobacterium ivanovi* on Sephacryl S-300. Molecular weight standards used: (1) thyroglobulin, 669,000; (2) ferritin, 440,000; and (3) aldolase, 158,000. GS is indicated by the arrow.

Purified *Methanobacterium ivanovi* GS was tested for inhibition by several feedback inhibitors in comparison with the value published for GS isolated from different eubacterial sources (Table 2). Glycine, L-alanine, L-tryptophan, L-histidine, and 5'-AMP significantly (>15%) inhibited the GS activity measured by the transferase reaction. Interestingly, glutamine (5 or 10 mM) alone did not inhibit the synthetic activity of the GS from *Methanobacterium ivanovi* as has been shown for the GS from *Bacillus subtilis* (17), but in the presence of AMP 85% inhibition of the GS activity in *B. subtilis* was observed, whereas the GS of *Methanobacterium ivanovi* remained unaffected.

L-Methionine-DL-sulfoximine, a glutamate analog and noncompetitive inhibitor of GS in many bacteria (23), strongly inhibited the GS in *Methanobacterium ivanovi*. L-Methionine-DL-sulfoximine (0.1 mM) inhibited the GS transferase activity by about 90%.

DISCUSSION

The GS of *Methanobacterium ivanovi* possesses a macromolecular structure similar to that of other procaryotic GSs (2, 28, 36). The GS is a dodecamer of about 600,000 daltons made up of a single type of subunit. It is interesting to note that although the enzymes of eucaryotic origin are octamers (28), all procaryotic GSs studied so far, including the present one from an archaebacterium, have a single type of subunit whose molecular weight falls in the range of 44,000 to 53,000. Thus, GSs have common features in both the archaebacterial and eubacterial kingdoms (19). Further studies on the amino acid sequencing of GS from different origins and DNA sequencing of *glnA* (the structural gene of GS) (14, 18, 27, 38) can give important indications of the degree to which the overall structure of the enzyme from different origins might have been conserved during evolution.

Although the GSs from different origins present structural similarities, their activities are regulated by different mechanisms (17, 26, 34, 35, 39, 40): divalent-cation-induced conformational changes; feedback inhibition by various end product metabolites; and covalent modification of the en-

TABLE 2. Comparison of feedback inhibitors of GS activity of *Methanobacterium ivanovi* with those reported for eubacterial enzyme

Inhibitor (concn [mM])	Inhibition of GS activity (%)		
	Detected value in *M. ivanovi*[a]	Published values in:	
		E. coli[a] (28)	*B. subtilis*[b] (17)
Control	0	0	0
L-Alanine (10)	65	30	75
Glycine (10)	38	10	51
L-Tryptophan (5)	30	15	6
L-Histidine (5)	17	9	16
5'-AMP (10)	23	30	95

[a] Transferase activity with Mn^{2+} as cofactor.
[b] Biosynthetic activity with Mg^{2+} as cofactor.

zyme subunits by a highly regulated reversible adenylylation-deadenylylation system (13, 21, 35, 39, 40). Fully adenylylated enzyme is physiologically inactive. Phosphodiesterase treatment releases the AMP group(s) and the enzyme becomes fully active. In general, the GSs from gram-negative bacteria (37, 40) are covalently modified, possibly by adenylylation; in contrast, no evidence of GS regulation by adenylylation could be found in gram-positive bacteria (37, 40), *Anabaena* sp. strain 7120 (26), yeasts (28), and also in the archaebacterium *Methanobacterium ivanovi*. In gram-negative bacteria such as *E. coli*, GS is subjected to adenylylation with a cascade control mechanism (1, 40) and is thus less affected by feedback inhibitors than GS from gram-positive bacteria. It has been proposed for the *B. subtilis* (17) and *Anabaena* sp. strain 7120 (26) enzymes that a stronger response to feedback inhibitors is necessary because both organisms lack the cascade modification system of *E. coli*. The strong inhibition of *Methanobacterium ivanovi* GS by alanine, glycine, and tryptophan can be explained similarly. Consequently, *Methanobacterium ivanovi* GS appears to be regulated similarly to that in gram-positive bacteria and *Anabaena* spp.

No antigentic cross-reactivity could be detected between the GS of *Methanobacterium ivanovi* and the antiserum to this enzyme from *E. coli*, *Anabaena* strain 7120, or *B. megaterium*. Tronick et al. (37) did not find a precipitin band between the crude extracts of *Methanosarcina barkeri* and the antiserum against *E. coli* GS. On the basis of a survey by Tronick et al. (37), *E. coli* GS antiserum showed antigenic homology with the GSs of a number of gram-negative bacteria, but no antigenic cross-reactivity was observed with the GSs of gram-positive bacteria or higher organisms. The only exception to these studies was the cross-reaction detected between *E. coli* GS antiserum and the gram-positive *Streptomyces* GS. Orr et al. (27) present a different view on the basis of the amino acid composition, NH_2-terminal sequences, and predicted conformation potentials of the GSs from *Anabaena* sp. strain 7120, a gram-negative bacterium (*E. coli*), and a gram-positive bacterium (*B. subtilis*). They demonstrated limited homology among all three enzymes, proposing that antigenic cross-reactivity does not necessarily depend upon the Gram reaction or state of adenylylation.

In general, the archaebacterial GS from *Methanobacterium ivanovi* appears similar in structure to the eubacterial GS, although it functionally resembles the GS from gram-positive bacteria rather than that from gram-negative bacteria. Further information on the functional and structural similarities may be provided by cloning *glnA* of *Methanobacterium ivanovi* in a *Bacillus* deletion mutant or *E. coli*.

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