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Ammonia Assimilation and Synthesis of Alanine, Aspartate, and Glutamate in *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum*

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The mechanism of ammonia assimilation in *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum* was documented by analysis of enzyme activities, $^{13}NH_3$ incorporation studies, and comparison of growth and enzyme activity levels in continuous culture. Glutamate accounted for 65 and 52% of the total amino acids in the soluble pools of *M. barkeri* and *M. thermoautotrophicum*. Both organisms contained significant activities of glutamine synthetase, glutamate synthase, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase. Hydrogen-reduced deazaflavin-factor 420 or flavin mononucleotide but not NAD, NADP, or ferredoxin was used as the electron donor for glutamate synthase in *M. barkeri*. Glutamate dehydrogenase activity was not detected in either organism, but alanine dehydrogenase activity was present in *M. thermoautotrophicum*. The in vivo activity of the glutamine synthetase was verified in *M. thermoautotrophicum* by analysis of $^{13}NH_3$ incorporation into glutamine, glutamate, and alanine. Alanine dehydrogenase and glutamine synthetase activity varied in response to $[NH_4^+]$ when *M. thermoautotrophicum* was cultured in a chemostat with cysteine as the sulfur source. Alanine dehydrogenase activity and growth yield (grams of cells/mole of methane) were highest when the organism was cultured with excess ammonia, whereas growth yield was lower and glutamine synthetase was maximal when ammonia was limiting.

Amino acids play an important role in the carbon and nitrogen metabolism of autotrophic methanogens. Alanine, glutamate, and aspartate were among the first readily identifiable anabolic intermediates in short-term $^{14}CO_2$ fixation experiments conducted with *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* (8). Patterns of acetate incorporation during growth (10, 39) and the analysis of tricarboxylic acid cycle enzymes (39, 43) indicated that these amino acids are derived from α-keto acid precursors.

M. thermoautotrophicum and *M. barkeri* are capable of autotrophic growth with CO_2 as the sole source of carbon and ammonia as the sole source of nitrogen (38, 45). The nitrogen requirements of *Methanobacterium ruminantium* and *Methanobacterium* strain M.O.H. were investigated by Bryant et al. (7); these species required ammonia as the source of nitrogen, with relatively little nitrogen being incorporated from amino acids supplied to the organism (7). The biochemical mechanism for ammonia assimilation has not been reported in methanogens (4, 41).

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The most common pathways of ammonia assimilation in microbes involve the formation of glutamate from ammonia and α-ketoglutarate (6). Either glutamate dehydrogenase (GDH) or glutamate synthase (GOGAT) and glutamine synthetase (GS) function in this regard. The other cellular amino acids are formed by transamination reactions using glutamate as the amino donor.

GDH, by virtue of its characteristically high K_m for ammonia, has been suggested to function in ammonia assimilation primarily at high levels of ammonia, and GS plus GOGAT have been found to do so at lower levels of ammonia (6). These two ammonia-assimilating systems have been detected in many organisms, including *Selenomonas*, *Nostoc*, *Aerobacter*, and *Anabaena* (20, 24, 25, 32).

The role of alanine dehydrogenase (ADH) in many organisms which contain the activity is in alanine catabolism or in sporulation (6). However, Aharonowitz and Friedrich (1) have provided evidence for the induction of the enzyme by ammonia or alanine in *Streptomyces clavuligerus*. A role in ammonia assimilation has been assigned to ADH in *S. clavuligerus* (1) and has been suggested for *Rhodopseudomonas capsu-*

lata (14), *Anabaena cylindrica* (29), and *Cyanidium caldarium* (27). The role of ADH in other organisms such as *Desulfovibrio desulfuricans* (11), *halobacterium salinarium* (16), and *H. cutirubrum* (17) is uncertain.

This study was undertaken to (i) elucidate the mechanisms used by methanogenic bacteria for the synthesis of alanine, aspartate, and glutamate and (ii) identify the reactions responsible for the assimilation of ammonia. Ammonia is used throughout this paper to indicate the assimilated substrate (NH_3 or NH_4^+). *M. thermoautotrophicum* and *M. barkeri* were chosen as model organisms for this study since amino acids play an important role in their anabolic metabolism (8) and they are representatives of the two major subdivisions of the methanogenic bacteria (4).

(A preliminary report of this work has appeared [W. R. Kenealy, P. J. Weimer, and J. G. Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, K57, p. 155].)

MATERIALS AND METHODS

Chemicals and gases. Substrates for enzyme assays and coupling enzymes were obtained from Sigma Chemical Company, St. Louis, Mo. All other chemicals were of reagent grade. Gases and gas mixtures were purchased from Matheson Scientific, Inc., Joliet, Ill. Traces of oxygen were removed from the gases by passage through heated copper filings. [*U*-^{14}C]α-ketoglutarate (150 to 200 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. Factor 420 (F_{420}) was purified from *M. barkeri* according to the procedure of Eirich et al. (9).

Organisms and cultivation. Stock cultures of *M. thermoautotrophicum* strain ΔH and *M. barkeri* strain MS were maintained as described previously (15). *M. barkeri* and *M. thermoautotrophicum* were mass cultured with H_2/CO_2 (80:20) in a 14-liter New Brunswick Microferm fermenter as described by Weimer and Zeikus (38) and Zeikus et al. (43). *M. barkeri* was mass cultured on methanol in 20-liter carboys as described by Weimer and Zeikus (38). Cells were harvested anaerobically in a Sorvall RC-5 centrifuge equipped with a KSB continuous-flow system.

Cultures of *M. thermoautotrophicum* were grown with limiting and excess ammonia concentrations in a New Brunswick Multigen continuous-culture fermenter. The reservoir contained a low-phosphate-buffered mineral medium (15) with the concentration of NH_4Cl changed to limit the growth of the organism. The medium was reduced with cysteine-hydrochloride at a final concentration of 2 mM. Sodium sulfide was not used as a reducing agent or sulfur source because it was too easily removed from the medium by continuous gassing. The working volume of the vessel was 265 ml, and a constant gassing rate of 150 cm^3/min for H_2/CO_2 (80:20) was used. Once the culture reached the steady state, the chemostat effluent was collected anaerobically at 0 to 4°C. The effluent was centrifuged at 20,000 × *g* in stainless-steel bottles which were filled in an anaerobic glove box (Coy Products, Ann Arbor, Mich.). The cell pellets were suspended in an equal volume of anaerobic distilled water and stored as a frozen suspension at −20°C.

Metabolic analyses. Growth of *M. thermoautotrophicum* was measured by optical density at 660 nm in a Spectronic 20 (Bausch & Lomb, Rochester, N.Y.). The methane content of the chemostat outlet gas was quantified with a Varian Aerograph 600D gas chromatograph as described by Zeikus et al. (44). The ammonium content of the culture fluid was measured by a modification of the indophenol method of McCullough (23). To 5 ml of sample the following were added with mixing: 0.2 ml of 10% phenol in 95% ethanol; 0.2 ml of 5% nitroprusside; and 0.5 ml of a 4:1 mix of 20% sodium citrate plus 5% sodium hydroxide-Clorox bleach. After standing for 1 h, the absorbance at 640 nm was measured, and the [NH_4^+] was calculated from a standard curve.

Intracellular amino acids were extracted from 500 mg of freeze-dried logarithmic-phase H_2/CO_2-grown cells with 15 ml of 50% ethanol and brought to dryness on a Buchi rotary evaporator (Brinkmann Instruments Inc., Westbury, N.Y.). The dried extract was suspended in distilled water, and soluble proteins were precipitated by the addition of sulfosalicyclic acid to a final concentration of 5%. The amino acid content of the samples was determined by the amino acid analysis facility, Biophysics Laboratory, University of Wisconsin, Madison.

Preparation of cell extracts and measurement of enzyme activities. Anaerobic conditions were maintained throughout the procedure by gassing all experimental equipment with hydrogen. Cells were suspended in an equal volume of distilled water containing a final concentration of 2 mM $MgCl_2$ and 2 mM dithiothreitol. The suspension was passed through a French pressure cell at 48,300 KN/m^2. The lysate was collected in a Spinco centrifuge tube (Beckman Instruments, Inc., Lincolnwood, Ill.) sealed with a rubber bung. The lysate was centrifuged at 30,000 × *g* at 4°C for 20 min. The supernatant fluid was removed by syringe, and cell extracts were used immediately or stored at −20°C under hydrogen in serum bottles sealed with black rubber bungs. The protein content of the extracts was determined by the method of Lowry et al. (21), using bovine serum albumin in 2 mM dithiothreitol as a standard.

All spectrophotometric enzyme assays were measured on an Eppendorf 1101 spectrophotometer equipped with a 334-nm filter and a constant-temperature cuvette holder. The assays were performed in anaerobic 1.4-ml cuvettes containing 1 ml of assay mixture as described by Zeikus et al. (43). Anaerobic conditions were maintained to minimize NADH oxidation catalyzed by extracts in air. All activities reported were determined under conditions in which they were linear with time and protein concentration.

ADH (EC 1.4.1.1) was measured by following the oxidation of NADH at 334 nm (ε_{334} = 6.1 nmol^{-1} cm^{-1}). The assay mixture contained: Tris-hydrochloride buffer, pH 8.0 to 8.3 (at temperature of incubation), 100 mM; NH_4Cl, 100 mM; sodium pyruvate, 5 mM; NADH, 0.3 mM; and 10 μl of cell extract (~0.2 mg of extract protein).

Glutamate oxaloacetate transaminase (EC 2.6.1.1.) was measured by coupling the reaction to malate dehydrogenase and following the oxidation of NADH at 334 nm. The reaction mixture contained: Tris-

hydrochloride buffer, pH 8.4 (40°C), 100 mM; sodium aspartate, 20 mM; sodium α-ketoglutarate, 5 mM; NADH, 0.3 mM; pyridoxal phosphate, 0.1 mM; malate dehydrogenase, 2.5 U; and 10 μl of cell extract. The assay was initiated by addition of α-ketoglutarate.

Glutamate pyruvate transaminase (EC 2.6.1.2) was assayed by coupling the reaction to lactate dehydrogenase. The reaction mixture contained: Na_2CO_3 buffer, pH 8.95 (40°C), 100 mM; alanine, 60 mM; sodium α-ketoglutarate, 5 mM; NADH, 0.3 mM; pyridoxal phosphate, 0.1 mM; lactate dehydrogenase, 4.3 U; and 10 μl of cell extract. The assay was initiated by addition of α-ketoglutarate.

GS (EC 6.3.1.2) was measured by the γ-glutamyl hydroxamate exchange assay of Shapiro and Stadtman (31) or the synthesis assay of Bender et al. (5). The assay was initiated by addition of extract, and background controls lacked glutamine for the exchange assay and ATP for the synthesis assay.

GDH (EC 1.4.1.2) was assayed by following the oxidation of NAD(P)H at 334 nm. The reaction mixture contained: Tris-hydrochloride buffer, pH 8, 100 mM; NAD(P)H, 0.3 mM; NH_4Cl, 80 mM; sodium α-ketoglutarate, 5 mM; and 10 μl of cell extract. Pyridine nucleotide-linked GOGAT (EC 1.4.1.13, EC 1.4.1.14) was assayed as for GDH with 5 mM glutamine substituted for 80 mM NH_4Cl.

GOGAT (EC 1.4.99) was measured by a modification of the method of Lea and Mifflin (20). The conversion of [^{14}C]α-ketoglutarate to glutamate was determined by quantification of [^{14}C]glutamate eluted from Dowex AGlx8 acetate resin (Bio-Rad Laboratories, Richmond, Calif). Samples were centrifuged (Beckman Microfuge; Beckman Instruments, Inc., Anaheim, Calif.), and 0.5 ml of supernatant was applied to columns (40 by 10 mm) (Econo columns; Bio-Rad) with a 2-ml resin bed previously equilibrated with distilled water. The columns were eluted with 9.5 ml of distilled water and then 10 ml of 0.5 N acetic acid. Samples of the acetic acid eluant were counted in 5 ml of Instagel (Packard Instruments Co. Inc., Rockville, Md.) in a Packard Prias PLD scintillation counter. Glutamate was the only labeled product present in the acetic acid eluant after incubation of [^{14}C]α-ketoglutarate with extracts, as evidenced by comigration with glutamate in the thin-layer electrophoresis–thin-layer chromatography (TLC)–autoradiography procedure of Daniels and Zeikus (8). Extracts where indicated were pretreated with DEAE-52 (Whatman)–cellulose to remove anionic compounds such as F_{420} or ferredoxin as described by Lamed and Zeikus (18).

$^{13}NH_4^+$ incorporation studies. Time course incorporation studies with ^{13}N ammonia were conducted at the Michigan State University Heavy Ion Laboratory, East Lansing. $^{13}NH_4^+$ was generated as described by Thomas et al. (36) and made anaerobic by the gassing with N_2 and addition of sodium sulfide. *M. thermoautotrophicum* was grown on H_2/CO_2 in pressure tubes and harvested in the mid-log phase of growth by centrifugation. The cell pellet (2.8 mg) was suspended in anaerobic tubes that contained ammonium-free medium (4 ml), a H_2/CO_2 head space, and approximately 6 mCi of $^{13}NH_4^+$. Samples were removed at different times after the addition of label, extracted with an equal volume of ethanol, and treated as described by Wolk et al. (40). The cell residue was removed by centrifugation, and the supernatant fluid was dried under vacuum at 50°C. After suspension with methanol, standard amino acids were added, and the concentrated extract was applied to glass-backed, cellulose (0.1-mm coating) TLC plates (E. Merck AG, Darmstadt, West Germany) and electrophoresed on a 011 SAE 3202 high-voltage electrophoresis apparatus (Shandon Scientific Co., London, England) for 10 min at 3 kV. The radioactivity in the labeled intermediates was detected by a 7201 radiochromatogram scanner (Packard). The percentage of radioactivity was determined for glutamate, glutamine, and alanine by cutting out the corresponding area on the scan tracing. All weights determined were corrected for the decay which took place during the scanning by using the half-life value of 10 min and scan rate of 2 cm/min. Amino acid positions on the TLC plates were confirmed by spraying with ninhydrin.

RESULTS

Table 1 shows the quantity and type of amino acids present in the soluble pool of H_2/CO_2-grown *M. thermoautotrophicum* and *M. barkeri*.

TABLE 1. Intracellular amounts of amino acids in methanogens[a]

Amino acid	*M. thermoautotrophicum*		*M. barkeri*	
	μmol/500 mg of cells	% of amino acids	μmol/500 mg of cells	% of amino acids
Aspartate	1.81 ± 0.24	2.5	2.50 ± 0.54	3.1
Threonine	0.85 ± 0.12	1.2	1.96 ± 0.42	2.4
Serine	0.65 ± 0.16	0.9	0.49 ± 0.23	0.6
Glutamate	37.86 ± 4.92	51.5	53.04 ± 12.27	64.8
Glutamine	Present		Present	
Proline	0.89 ± 0.09	1.2	0.81 ± 0.35	1.0
Glycine	3.88 ± 0.50	5.3	4.12 ± 0.89	5.0
Alanine	23.73 ± 2.55	32.3	15.25 ± 1.66	18.6
Valine	0.75 ± 0.24	1.0	1.48 ± 0.28	1.8
Leucine	0.58 ± 0.10	0.8	0.70 ± 0.10	0.9
Lysine	1.80 ± 0.28	2.4	0.95 ± 0.20	1.2
Arginine	0.67 ± 0.13	0.9	0.53 ± 0.12	0.6
Total	73.47		81.83	

[a] Cells were extracted and analyzed as described in Materials and Methods. The values represent the mean ± standard deviation for duplicate analyses of two different batches of cells.

TABLE 2. Comparison of ammonia transformation enzyme activities in methanogen cell extracts[a]

Enzyme	Sp act (nmol/min per mg of protein)	
	M. thermoautotrophicum[b]	*M. barkeri*
GS	6.1 ± 2.6	93.0 ± 25.8
GDH	<0.05	<0.05
GOGAT	<0.05	<0.05
Glutamate pyruvate transaminase	102.0 ± 25.9	6.4 ± 1.19
Glutamate oxaloacetate transaminase	348.8 ± 124.2	9.7 ± 2.69
ADH	15.7 ± 4.5	<0.05

[a] Extracts were prepared and enzyme activities were assayed as described in Materials and Methods. Values represent the mean ± standard deviation for enzyme activity in many different cell extracts. Values represent activity at 60 and 37°C for *M. thermoautotrophicum* and *M. barkeri*, respectively. GS and GDH were measured by the pyridine nucleotide-linked assay. Glutamine synthetase was measured by the synthesis assay for *M. thermoautotrophicum* and by the exchange assay for *M. barkeri*.

[b] Activities of glutamate pyruvate transaminase and glutamate oxaloacetate transaminase were measured at 40°C and calculated for 60°C by the Q_{10} relation of 1.52 and 1.54, respectively.

Glutamate was present in both organisms and was the most abundant amino acid. It accounted for 64.8 and 51.5% of the amino acids of *M. barkeri* and *M. thermoautotrophicum*, respectively. *M. barkeri* contained less alanine in its soluble pool than did *M. thermoautotrophicum*. The other amino acids comprised less than 20% of the total amino acids and were present in similar amounts in both organisms. Glutamine was also detected in the soluble pool of both organisms, but the extraction procedure did not allow a meaningful quantification of the amount present.

The enzymes of ammonia assimilation and the transaminases between glutamate and pyruvate or oxaloacetate were assayed in extracts of *M. thermoautotrophicum* and *M. barkeri* (Table 2). The extracts were prepared from cells grown with an average doubling time of ~10 to 12 h. No differences in enzyme activity were detected for *M. barkeri* cells grown with H_2/CO_2 or methanol as the energy source.

GS activity was detected in both organisms by the γ-glutamyl hydroxamate synthesis or exchange assay. The value reported in Table 2 for *M. thermoautotrophicum* was that determined by the synthesis assay. The exchange assay gave 3.5-fold higher activities for *M. barkeri*, so it was used to determine the GS activity. The activity from both organisms was not sensitive to oxygen. *M. thermoautotrophicum* GS activity was not affected by 20 mM glutamine, which is the concentration used in the exchange assay. Attempts to demonstrate either a GDH or a GOGAT activity, which was dependent on pyridine nucleotides, in either methanogen were unsuccessful. Addition of *M. barkeri* or *M. thermoautotrophicum* extracts to control assays with *Escherichia coli* extracts did not alter the activity of its GOGAT or GDH.

Both methanogens had detectable levels of the transaminases between glutamate and pyruvate or oxaloacetate. The transaminase activities of *M. thermoautotrophicum* were much higher than those determined for *M. barkeri* and were stimulated 1.5-fold by the addition of pyridoxal phosphate. This stimulation was not observed for *M. barkeri*. The transaminase activities were completely inhibited by 5 mM aminooxyacetate and were not sensitive to oxygen.

ADH activity was detected in extracts of *M. thermoautotrophicum* but not *M. barkeri*. Addition of *M. barkeri* extract did not inhibit the ADH activity of *M. thermoautotrophicum*. *M. thermoautotrophicum* ADH was dependent on pyruvate, NADH, and ammonia. NADPH also served as a reductant, but the activity measured was less than 30% of the NADH value.

Figure 1 shows the effect of NH_4Cl concentra-

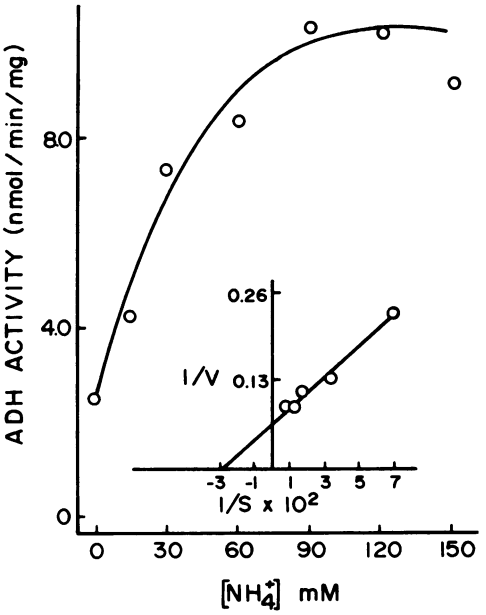

FIG. 1. Effect of NH_4^+ concentration on ADH activity in cell extracts of *M. thermoautotrophicum*. The assay conditions were: 100 mM Tris-hydrochloride, pH 8.0 to 8.3; 5 mM sodium pyruvate; 0.3 mM NADH; and variable NH_4Cl. The assay was performed at 40°C under an argon gas phase. The points represent the average of triplicate determinations.

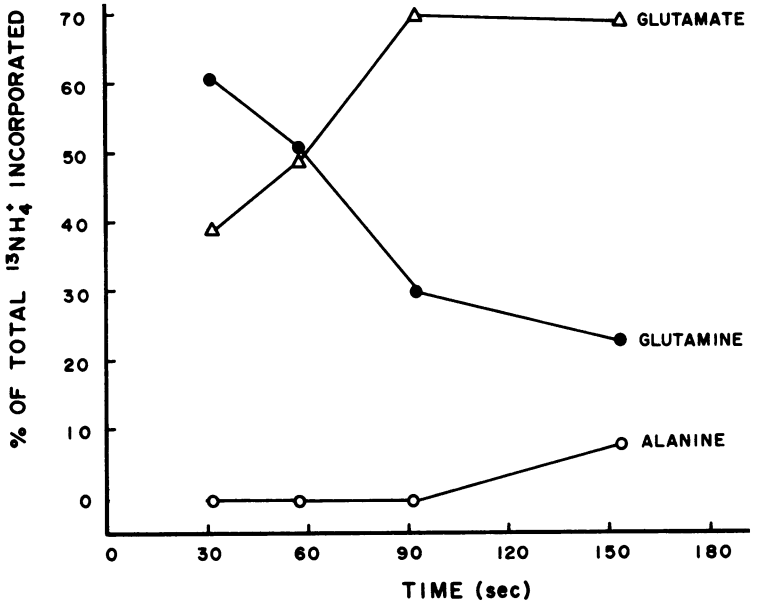

FIG. 2. Time course of $^{13}NH_4^+$ assimilation by cell suspensions of *M. thermoautotrophicum*. Cells were grown on H_2/CO_2, exposed to label, and analyzed as described in Materials and Methods.

tion on the ADH activity of *M. thermoautotrophicum*. The [NH_4Cl] of half-maximal velocity for ADH was 37 mM as calculated from the reciprocal plot. Values for pyruvate and NADH calculated from similar plots were 1 and 0.01 mM, respectively. Arrhenius plots of the log ADH activity versus 1/°K were linear from 30 to 70°C and displayed a Q_{10} relation of 1.40. The ADH activity reported in Table 2 is for the aminating direction. The deaminating activity of ADH could be demonstrated at pH $\geq$ 10.0; however, a nonenzymatic reduction of NAD by alanine was observed at pH 10.5. The rate of the deamination was much less than that for the forward reaction. The ADH activity was not sensitive to oxygen.

Figure 2 shows a time course for $^{13}NH_4^+$ incorporation into amino acids of *M. thermoautotrophicum* cell suspensions under conditions of low [NH_4^+] (~0.075 mM). At the first sampling point, glutamine was labeled to a greater extent than was glutamate. The percentage of label in glutamine decreased with time as glutamate increased. Alanine became labeled only upon extended incubation. Subsequent repeats of this experiment showed nearly equal amounts of label in glutamate and glutamine at early times. The percentage of label in glutamate increased with time as the percentage of label in glutamine decreased. Time course experiments where 10 mM NH_4Cl was included resulted in too little incorporation for analysis.

Since in vivo labeling experiments supported the presence of GOGAT activity, a new approach was used for assaying this enzyme in cell extracts. Table 3 presents the results of these

TABLE 3. Glutamate formation activity in methanogen cell extracts[a]

Assay system	Sp act (nmol/min per mg of protein)	
	M. thermoautotrophicum	*M. barkeri*
Complete	15.6	60.4
− Glutamine	11.5	26.5
− H_2	15.5	28.1
− Methyl viologen	14.5	48.5
Complete + aminooxyacetate	0.0	36.1
− Glutamine	0.0	10.8
− H_2	0.8	1.3
− Methyl viologen	0.0	21.6

[a] The complete assay contained: 100 mM Tris-hydrochloride buffer, pH 8, 5 mM [^{14}C]α-ketoglutarate (44.8 dpm/nmol), 5 mM methyl viologen, 2 mM glutamine, and a hydrogen gas phase. Concentrations of additions were: 5 mM aminooxyacetate or 1 atm of N_2 in lieu of H_2. The total reaction volume was 1 ml, and the reaction was initiated with 100 μl of extract which was stabilized with 2 mM α-ketoglutarate. The reaction was terminated with 1 ml of methanol after 10 min of incubation at 37 or 60°C for *M. barkeri* or *M. thermoautotrophicum*, respectively. Activities reported are averages of duplicate determinations and are representative of many experiments.

TABLE 4. Specificity of electron donors for GOGAT of *M. barkeri*

Conditions	GOGAT activity (nmol of glutamate formed/min per mg of protein)
Standard conditions[a]	0.3
− Hydrogen	0.1
+ 0.04 mg of spinach ferredoxin/ml	0.3
+2.5 mM flavin adenine dinucleotide	0.9
+ 2.5 mM flavin mononucleotide	4.4
+ 2.5 mM NAD	0.5
+ 2.5 mM NADP	0.4
+ *M. barkeri* F_{420}[b]	5.1
+ 5 mM methyl viologen	10.2
− Hydrogen + 2.5 mM NADH	0.4
− Hydrogen + 2.5 mM NADPH	0.3

[a] Standard conditions were: 100 mM Tris-hydrochloride buffer, pH 8, 5 mM aminooxyacetate, 2 mM glutamine, 5 mM [^{14}C]α-ketoglutarate (44 dpm/nmol), and a hydrogen gas phase in a total volume of 1 ml. DEAE-52-cellulose-treated extract (0.1 ml) was added to initiate the reaction, which was terminated at 10 min by the addition of 1 ml of methanol. Activities reported are the average of duplicate determinations.

[b] F_{420} concentration = 50 μl of a solution with an absorbance at 420 nm of 1.8 at pH 7.

experiments using the conversion of [^{14}C]α-ketoglutarate to [^{14}C]glutamate. Glutamate was identified as the sole labeled product of the reaction by thin-layer electrophoresis-TLC and autoradiography. The GOGAT activity of *M. barkeri* was dependent on glutamine and hydrogen and was stimulated by addition of methyl viologen. The addition of NH_4Cl and MgATP did not replace glutamine in the reaction, although there was some stimulation of glutamate synthesis with the additions. The GOGAT activity of *M. thermoautotrophicum* was dependent on glutamine. The absence of hydrogen or methyl viologen did not decrease the activity as with *M. barkeri*. The addition of aminooxyacetate, an inhibitor of aminotransferases, significantly inhibited the GOGAT activity of *M. thermoautotrophicum* and removed background activity in both organisms. Cell extracts of *M. thermoautotrophicum* but not *M. barkeri* lost all GOGAT activity after anaerobic dialysis to remove low-molecular-weight metabolites. The inclusion of dithionite in the assay for either organism caused an inhibition of glutamate formation. The extracts contained a very active hydrogenase which turned the methyl viologen blue immediately upon addition of extract.

The hydrogen dependence of the activity was further investigated in extracts of *M. barkeri*. Removal of soluble anionic compounds by treatment with Whatman DEAE-52-cellulose resulted in loss of hydrogen-dependent activity (Table 4). The addition of flavin mononucleotide, *M. barkeri* deazaflavin-F_{420}, or methyl viologen restored the hydrogen-dependent activity. Neither the addition of spinach ferredoxin, flavin adenine dinucleotide, NAD, or NADP nor the addition of NADH or NADPH in the absence of hydrogen stimulated glutamate synthesis. In experiments using untreated extracts, F_{420} or methyl viologen addition stimulated the activity over that of hydrogen alone.

Experiments were initiated to limit the growth of *M. thermoautotrophicum* with medium [NH_4^+]. Figure 3 illustrates the growth response of *M. thermoautotrophicum* to [NH_4^+] in batch cultures which contained Na_2S as the sole sulfur source. Growth yield but not growth rate was directly influenced by increasing NH_4Cl. Neither growth rate nor final yield was inhibited by 200 mM NH_4Cl. A yield of 0.121 mg of cells/

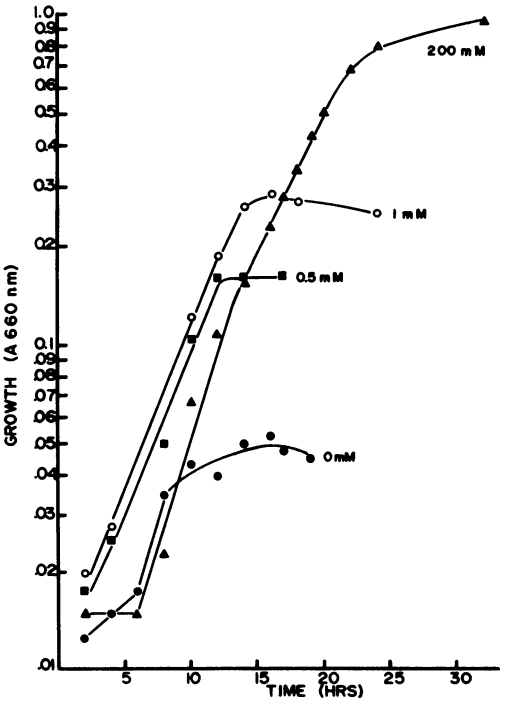

FIG. 3. Effect of [NH_4^+] on growth of *M. thermoautotrophicum* in batch culture. Cells were grown in pressure tubes containing 5 ml of mineral medium, with Na_2S as the sole sulfur source and the indicated concentration of added NH_4Cl. The culture head space was maintained at greater than 1 atm of H_2/CO_2 during the experimental time course. The points represent the average of duplicate tubes.

μmol of NH_4Cl was calculated from the optical density at 660 nm values obtained after growth on 0.5 mM and 1 mM NH_4Cl with the 0 mM NH_4Cl value subtracted. The dry weight of cells per liter of culture for an optical density of 1.0 was 550 mg.

M. thermoautotrophicum was cultured in a chemostat to determine the effect of ammonia concentrations on growth and ammonia-assimilating enzyme activities (Table 5). When the organism was shifted from excess ammonia to a condition where growth was limited by ammonia, the specific activity of ADH decreased and that of GS increased. When the reverse experiment was performed, ADH activity increased and GS activity decreased in response to the shift from limiting to excess ammonia concentrations. *M. thermoautotrophicum* removed almost all of the NH_4^+ from the medium in the first experiment and was also limited by ammonia in the second experiment, since the optical density increased upon addition of more NH_4Cl. The yield of cells per mole of methane produced paralleled the increase and decrease of ADH specific activity. Yield values of 0.142 to 0.215 mg of cells/μmol of NH_4^+ used were calculated for ammonia-limited growth in a chemostat.

DISCUSSION

The data indicate that methanogens contain the same mechanism for ammonia assimilation as do other bacteria, namely, GS/GOGAT. In addition, *M. thermoautotrophicum* contains ADH activity as a second mechanism of ammonia assimilation, which is unusual since most bacteria contain GDH as a second system of assimilation. Glutamate and alanine comprised the majority of the amino acids in the soluble pool for both *M. thermoautotrophicum* and *M. barkeri*. The high levels of glutamate in both organisms suggest that glutamate is central to the formation of other amino acids via transaminases. Since the pool sizes of the amino acids vary drastically only with the ionic strength and not sources of limitation of growth (35), the high levels of alanine and glutamate would indicate that they are involved in primary ammonia assimilation as suggested for *Bacillus* sp. (3).

The level of ADH activity in *M. thermoautotrophicum* grown in continuous culture was regulated by the concentration of ammonia in the medium. ADH in *M. thermoautotrophicum* appears to function in ammonia assimilation like GDH in most other bacteria. The inverse relationship between the amino acid dehydrogenases and the GS and GOGAT levels can be attributed to the differences in the K_m of NH_4^+ for GS and GDH or ADH (6, 13). GDH is used at high levels of ammonia, and GS and GOGAT are used at low levels of ammonia (6, 25, 28, 32). When *M. thermoautotrophicum* was grown with limiting ammonia, the growth yield was lower than under conditions of energy limitation. This was also seen for *Aerobacter aerogenes* grown with glycerol or ammonia as the limiting substrate (34). When cells use GS/GOGAT to assimilate NH_4^+, the cell yield is lower because of the ATP requirement for the pathway (33).

The environments from which *M. thermoautotrophicum* has been isolated (42, 45) are all high in ammonia. Washburn Hot Springs in Yellowstone contains 34 mM NH_4^+ (J. G. Zeikus, unpublished data), and sewage digesters contain levels of ammonia as high as 100 to 200 mM (21). Ammonium concentrations of ≥200 mM inhibit methane production in anaerobic digestors (22). The growth rate of *M. thermoautotrophicum* is unaffected by 200 mM NH_4Cl. In light of these results, digester failure caused by high ammonia concentrations may not be due to inhibition of methanogens per se.

TABLE 5. Effect of ammonium concentration on the specific activities of ADH and GS in *M. thermoautotrophicum* grown in continuous culture[a]

Response to ammonium	$[NH_4^+]$ (mM)		Growth[b]	Growth yield (g of cells/mol of CH_4)	Doubling time (h)	Sp act[c] (nmol/min per mg of protein)	
	Reservoir	Vessel				ADH	GS
I. Initial excess	15.4	13.2	0.443	2.26 ± 0.17	3.90	2.96 ± 1.26	0.78 ± 0.35
Limited	1.5	0.02	0.381	2.15 ± 0.22	4.05	0.49 ± 0.35	1.54 ± 0.64
II. Initial limitation	1.5	0.88	0.242	1.87 ± 0.09	2.60	0.56 ± 0.44	1.43 ± 0.71
Excess	20.0	ND	0.448	2.18 ± 0.10	2.60	1.98 ± 0.65	0.86 ± 0.31

[a] Cells were grown in a chemostat and were harvested as described in Materials and Methods. GS activity was determined by the γ-glutamyl synthesis assay. ADH activity, ammonium concentration, and growth were measured as described in Materials and Methods. ND, Not determined.

[b] Determined as optical density at 660 nm.

[c] Activities reported are means ± standard deviation for >10 determinations of each activity at 60°C.

The levels of ADH and GS were higher in batch cultures than in the chemostat cultures. The medium used in each of the experiments differed only in the sulfur source supplied. The batch cultures were supplied sulfide as the sole sulfur source, where as the chemostat cultures only received cysteine. This indicates that *M. thermoautotrophicum* may use either as its sulfur source, an observation not seen with *M. barkeri* cultures (30). If the nitrogen present in cysteine were also incorporated, this would decrease the requirement of the cells for ammonia and therefore lessen the ammonia assimilation activity required. Batch cultures had a lower yield of cells per micromole of NH_4^+ assimilated than was observed in the chemostat. Assuming the nitrogen content of the cell to be the same (NH_4^+ -limiting growth in the chemostat and in batch culture), the different yields would indicate that cysteine supplies some of the nitrogen to the organism. Further studies are needed to indicate if cysteine can also be used as the sole sulfur and nitrogen source.

The labeling studies with $^{13}NH_4^+$ in *M. thermoautotrophicum* indicated that ammonia was assimilated by the GS/GOGAT pathway and not by ADH. The high K_m for ammonia of ADH would indicate that the enzyme is functional only at higher levels of ammonia. The yield of $^{13}NH_3$ obtained by Thomas et al. (36) was approximately 0.3 μmol, which when diluted to 4 ml would give an ammonia concentration of 0.075 mM. Consequently, ADH activity would not be detected with these conditions.

Both *M. thermoautotrophicum* and *M. barkeri* contained GS activity. The activity of *M. thermoautotrophicum* had to be measured by the synthesis assay. The exchange assay which was used for *M. barkeri* could not be used with *M. thermoautotrophicum* since it was not linear with time. The lack of the transferase activity has been noted in *Selenomonas ruminantium* (32) and in *Bacillus licheniformis* (12). In the latter organism, the lack of the transferase activity was presumed to be due to an inhibition of the activity by glutamine. Such an inhibition was not seen for *M. thermoautotrophicum* or *S. ruminantium* (32), so it remains unclear why these organisms do not exhibit the transferase activity.

The GOGAT activity of both *M. thermoautotrophicum* and *M. barkeri* could not be measured by the oxidation of NAD(P)H. This has been observed in many organisms, such as cyanobacteria (20), higher plants (19, 26), and *S. ruminantium* (32), and the assay is accomplished by measuring the [^{14}C]glutamate formation from [^{14}C]α-ketoglutarate. GOGAT activity in *M. thermoautotrophicum* was established by enzyme analysis and by the labeling pattern of the amino acids of the short-term $^{13}NH_4^+$ incorporation. The GOGAT activity in *M. barkeri* extracts was dependent on hydrogen addition. When the extract was treated to remove soluble electron donors, the activity was absolutely dependent on addition of electron carriers to mediate the transfer of electrons from hydrogen. The data presented suggests that flavin mononucleotide or deazaflavin-F_{420} may be the physiological electron donor in this reaction.

In sum, the data presented here suggest several significant differences between *M. thermoautotrophicum* and *M. barkeri*. In addition to the presence of ADH in *M. thermoautotrophicum*, other catalytic features of common enzymes appeared different (for example, notably higher GS and GOGAT activities but lower transaminase activities in *M. barkeri*). Perhaps other important differences in ammonia metabolism remain to be described. In this regard, only *M. barkeri* can grow on methylamine as the sole carbon and nitrogen source (38).

Daniels and Zeikus (8) established that alanine, glutamate, and aspartate were the first identifiable products of autotrophic [^{14}C]CO_2 incorporation in *M. thermoautotrophicum* and *M. barkeri* and that these bacteria lacked the Calvin cycle or methylotrophic assimilation pathways of aerobes. Previous enzymatic and [^{14}C]acetate incorporation studies demonstrated that both species lacked a complete tricarboxylic acid cycle but synthesized α-ketoglutarate via different portions of this pathway (10, 39, 43). The present data provide enzymatic documentation for the α-keto acid amination to glutamate, alanine, and aspartate in these two phylogenetically diverse methanogens.

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