Solution ^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis of the Amino Acids of *Methanosphaera stadtmanae*: Biosynthesis and Origin of One-Carbon Units from Acetate and Carbon Dioxide

TERRY L. MILLER,^{1*} XI CHEN,^{2†} BIN YAN,² AND SHELTON BANK²

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201-0509,¹
and Department of Chemistry, State University of New York at Albany, Albany, New York 12222²

Received 7 November 1994/Accepted 7 January 1995

We found that general pathways for amino acid synthesis of *Methanosphaera stadtmanae*, a methanogen that forms CH_4 from H_2 and methanol, resembled those of methanogens that form CH_4 from CO_2 or from the methyl group of acetate. We determined the incorporation of ^{14}C -labeled CO_2 , formate, methanol, methionine, serine, and acetate into cell macromolecules. Labeling of amino acid carbons was determined by solution nuclear magnetic resonance spectroscopy after growth with ^{13}C -labeled acetate, CO_2 , serine, and methanol. The α and β carbons of serine and alanine were formed from carboxyl and methyl carbons of acetate, respectively, and the amino acid carboxyl groups were formed from CO_2 . This indicates that pyruvate was formed by reductive carboxylation of acetate. Labeling of the methyl carbon of methionine indicated that the major route of synthesis was from the hydroxymethyl carbon of serine that arises from the methyl carbon of acetate. Methanol was a minor source of the methyl of methionine. Unambiguous assignment was made of the sources of all carbons of histidine. Labeling of the histidine 7 position (ϵ carbon) was consistent with formation from the C-2 of the purine ring of ATP and the origin of the C-2 from a formyl unit derived from the hydroxymethyl carbon of serine.

The primary substrates used by methanogens for the production of CH_4 are H_2 plus CO_2 , formate, acetate, methanol, and methylamines (10). Almost all methanogens use H_2 to reduce CO_2 to CH_4 . *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*) species use acetate and form CH_4 from its methyl group rather than by CO_2 reduction. *Methanosarcina* species also form CH_4 from methanol and methylamines; they couple the oxidation of methyl groups to CO_2 to the reduction of methyl groups to CH_4 . *Methanosphaera stadtmanae* uses H_2 to reduce methanol to CH_4 . It cannot oxidize methanol to CO_2 (17, 18). *M. stadtmanae* is incapable of using any of the other known substrates for methanogenesis. It cannot use H_2 to reduce CO_2 to CH_4 , and it does not produce CH_4 from acetate.

M. stadtmanae synthesizes almost all of its cellular carbon from acetate and CO_2 (17, 18). This implies that it produces reduced one-carbon derivatives (formyl, hydroxymethyl, and methyl compounds) that are used for biosynthesis but not for CH_4 formation. *M. stadtmanae* is uniquely suitable for investigation of the interconversion of one-carbon biosynthetic intermediates by using specifically labeled ^{13}C acetate and $^{13}\text{CO}_2$ because all of the enriched carbon atoms are incorporated into cell carbon. Methanogens use one-carbon derivatives of unique coenzymes as substrates for enzymatic reduction of CO_2 to methane (11, 20). Jones et al. (9) proposed that the reduced one-carbon pterin intermediates also participate in biosynthetic one-carbon transfer reactions. Whether the same coenzymes and enzymes participate in the synthesis of

serine, glycine, methionine, thymine, and purines in *M. stadtmanae* has not been established.

Studies of ^{13}C acetate and $^{13}\text{CO}_2$ incorporation into amino acids of *Methanospirillum hungatei* and other methanogens provide clear evidence for the biosynthetic pathways used to synthesize amino acids, purines, and pyrimidines, including the pathways of reduced one-carbon biosynthesis (2–6, 22, 23, 25). All of the organisms studied reduce CO_2 to CH_4 or produce CH_4 from acetate. The present investigation was initiated to compare the biosynthetic pathways of *M. stadtmanae* with those of methanogens that form CH_4 from CO_2 or from the methyl group of acetate to determine whether there are differences in one-carbon biosynthetic routes. We determined the incorporation of ^{14}C -labeled CO_2 , formate, methanol, methionine, serine, and acetate into cell macromolecules. Labeling of amino acid carbons was determined in cells grown on ^{13}C -labeled acetate, CO_2 , serine, and methanol.

MATERIALS AND METHODS

Organism. *M. stadtmanae* MCB-3 (DSM 3091) was maintained by the liquid/solid (biphasic) culture technique (13) on methanol and H_2 in phosphate-buffered rumen fluid medium at -76°C as previously described (18). Cultures were transferred at 6-month intervals. Anaerobic conditions were maintained by using the serum bottle modification of the Hungate technique (16).

Growth and analysis of ^{14}C -labeled cells. A phosphate-buffered defined medium was used for ^{14}C -labeling studies. It contained (per liter) sodium acetate, 1 g; L-leucine, 0.13 g; L-isoleucine, 0.13 g; thiamine $\cdot$ HCl, 2 mg; biotin, 1 mg; D-pantothenic acid, 2 mg; nicotinamide, 2 mg; riboflavin, 2 mg; pyridoxine $\cdot$ HCl, 2 mg; cyanocobalamin, 20 μg ; p-aminobenzoic acid, 100 μg ; folic acid, 50 μg ; KH_2PO_4 , 2.8 g; K_2HPO_4 , 0.3 g; NH_4Cl , 2 g; $(\text{NH}_4)_2\text{SO}_4$, 0.3 g; NaCl, 0.61 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 150 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4.5 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 76 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 100 μg ; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8 mg; $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 180 μg ; H_3BO_3 , 100 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 100 μg ; Na_2SeO_4 , 1.9 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 92 μg ; nitrilotriacetic acid, 14 mg; resazurin, 1 mg; NaHCO_3 , 0.5 g; L-cysteine $\cdot$ HCl, 1.0 g; and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.5 g. The medium was dispensed and autoclaved under N_2 .

Anaerobic sterile methanol (final concentration, 0.16 M) and solutions of ^{14}C -labeled compound were added to the medium prior to its reduction with cysteine and sulfide. Acetate (12 mM) and bicarbonate/ CO_2 (400 μmol total)

* Corresponding author. Mailing address: Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza Box 509, Albany, NY 12201-0509. Phone: (518) 474-0015. Fax: (518) 474-8590.

† Present address: Department of Medicinal Chemistry and Pharmacognosy, Purdue University, Lafayette, IN 47907.

were present in all experiments. In separate experiments, the formate concentration was 5 mM and the serine and methionine concentrations were each 2 mM.

Media were inoculated (1%, vol/vol) with a culture in log growth phase ($A_{660} = 1$ to 2; $d = 1.8$ cm). The headspace of the bottles was replaced with 80% H_2 -20% CO_2 (2 atm [203 kPa]) after inoculation. Cultures (5 ml) were incubated at 37°C with rotation or shaking for 24 to 48 h. Portions of the headspace gas were removed to determine H_2 and CH_4 concentrations by gas chromatography (1).

Cells were collected by centrifugation, washed twice in 0.01 M potassium phosphate buffer (pH 7.0), and resuspended in distilled water. *M. stadtmannae* has a cell sacculus composed of pseudomurein (12) and does not lyse in distilled water. Portions of the washed-cell suspension were removed for determination of the total uptake of radioactive carbon into the cells. A standard curve relating dry weight of *M. stadtmannae* cells to optical density was used to estimate the weight of cells subjected to fractionation. The distribution of radioactive carbon in cellular components was determined by the trichloroacetic acid fractionation procedure of Roberts et al. (19). The carbon content of washed cells grown in the absence of radioactive carbon was determined in a total-carbon analyzer (model 0524B; Oceanography International Corp., College Station, Tex.).

The radioactivity of radioisotope solutions, suspended cells, and cellular fractions was determined in Aquasol (New England Nuclear, Boston, Mass.) in a scintillation counter (model 1217 Rackbeta; LKB Wallac, Turku, Finland). The radioactivity present in CO_2 was determined after injection of a known volume of gas into a closed vial containing phenethylamine. The radioactivity of portions of the phenethylamine was determined by using Aquasol. Radioactivity was corrected for quenching by the external-standard method as specified by the LKB Wallac procedures manual.

Growth of ^{13}C -labeled cells. All of the vitamins except biotin and thiamine were omitted from the medium described above, and the medium was modified for biphasic culture of the organism (18). Anaerobic biphasic cultures were prepared with a ratio of liquid to solid of 1:4. After autoclaving, sterile solutions of soluble 99% enriched ^{13}C -precursors were added to the liquid and solid phases prior to reduction of the medium. Methanol was present at 0.32 M in the solid phase and 0.16 M in the liquid phase. The gas phase was 2 atm of 80% H_2 -20% CO_2 . After ca. 20 h of incubation at 37°C, the headspace gas was replaced with H_2 - CO_2 twice daily. Cultures were harvested when the A_{660} ($d = 1.0$ cm) was between 4 and 6 (ca. 5 to 7 days). For studies of the incorporation of $^{13}CO_2$, the medium was prepared with 99% enriched [^{13}C]bicarbonate and an 80% H_2 -20% $^{13}CO_2$ atmosphere was prepared as described by Lajoie et al. (14). After 48 h, the culture headspace gas was replaced with 80% H_2 -20% $^{13}CO_2$ and the culture was incubated for an additional 48 h. The concentrations of ^{13}C -labeled soluble compounds in the liquid phase were as follows: 1- or 2-acetate, 6 mM; serine, 2 mM; and methanol, 160 mM. The solid phase contained the indicated compounds at twice the concentration as that in the liquid phase.

Protein hydrolysates. Cells were harvested, washed by centrifugation as described above, and freeze-dried. The protein was isolated from dried cells (25 to 50 mg) by the trichloroacetic acid fractionation procedure and hydrolyzed in 6 N HCl in vacuo at 108°C for 16 h. The hydrolysate was freeze-dried. The amino acid composition of a protein hydrolysate of cells grown in the absence of label was determined, as specified by the manufacturer, on a System Gold amino acid analyzer (Beckman Instruments Inc., San Ramon, Calif.).

NMR spectra of ^{13}C -labeled compounds. Nuclear magnetic resonance (NMR) spectra of ^{13}C -labeled compounds were acquired on a Varian XL-300 multinuclear spectrometer at 75.43 MHz, using broad-band proton decoupling, 20,000-Hz sweep width, and 20,032 data points. The pulse width was set at 11 μ s, corresponding to a 40° flip angle. The recycle time was 2.8 s, including 0.8 s of acquisition time and 2 s of delay time. A signal/noise ratio of high quality was obtained by using 3,000 to 30,000 transients. Chemical shifts were calibrated to the dioxane methylene peak at 67.4 ppm, which was referenced to tetramethylsilane.

Detection of amino acid carbon atoms. Dried protein hydrolysate (25 to 50 mg) labeled with $^{13}CO_2$, [^{13}C]acetate, [^{13}C]acetate, [^{13}C]serine, or [^{13}C]methanol or unlabeled hydrolysate (45 mg) was dissolved in 0.5 ml of D_2O with 5 μ l of 1,4-dioxane. The aqueous samples (pH < 2.0) were placed in 5-mm NMR tubes maintained at 25°C.

Analysis of the protein hydrolysate was based on the chemical shift differences between individual carbon atoms in 15 amino acids. A standard set of chemical shift values of unlabeled amino acids under the identical conditions of the protein hydrolysates was established. In addition, the protein hydrolysate of unlabeled cells provided a natural abundance background ^{13}C NMR spectrum. The chemical shift values in the unlabeled spectrum agreed with those of the standard set of amino acids.

The interpretation of spectra from hydrolysates other than that labeled with $^{13}CO_2$ was unambiguous. However, the spectrum for the hydrolysate labeled with $^{13}CO_2$ had more than 20 carboxyl carbon peaks gathered within a 3-ppm range, which precluded individual identification. Amino acids obtained from cells labeled with $^{13}CO_2$ were further separated by passage through a Dowex 1X8 (100/200 mesh) column eluted sequentially with water and HCl as described by Fuchs and Stupperich (7). The neutral and acidic fractions were dried in vacuo with P_2O_5 and NaOH. The amino acids in each dried fraction were separated by preparative chromatography on Whatman no. 1 paper with *sec*-butyl alcohol-

TABLE 1. Sources of cell carbon

^{14}C -labeled precursor	Cell dry wt (μ g)	Amt of cell carbon (μ g) ^a	Amt of ^{14}C incorporated (μ g)	% of ^{14}C incorporated ^b
Carbon dioxide	792	408	220	53.9
U-acetate	517	266	108	40.6
Formate	1,190	607	3.2	0.5
Methanol	525	268	2.4	0.9
Methyl, methionine	590	304	0.1	0.04
Hydroxymethyl, serine	540	278	2.3	0.8

^a Calculated from an experimentally determined value of 0.515 μ g of carbon per μ g (dry weight) of *M. stadtmannae* cells.

^b (Micrograms of isotopic carbon incorporated per microgram of cell carbon) $\times$ 100.

formic acid- H_2O (70:10:20) (19). Individual amino acids were eluted with water and freeze-dried. The dried residues were resuspended in D_2O and further prepared for analysis as described above.

Quantitative analysis. An estimation of the degree of enrichment of a specific amino acid carbon atom was based on two assumptions: (i) a fixed number of ^{13}C atoms in a similar environment have reproducible ^{13}C NMR signal intensity ratios under the same acquisition parameters with an adequate delay time, and (ii) the signal intensity ratio of a given peak to an internal-standard peak is directly proportional to the relative numbers of ^{13}C atoms that produce the signal. This hypothesis was tested with various concentrations of [^{13}C]glycine and a constant amount of dioxane. The plot of the [^{13}C]glycine/dioxane intensity ratios versus the concentration of glycine was linear. When cells are grown in the same defined medium at the same temperature, their amino acid composition is constant. The extent of enrichment of individual carbon atoms depends on the amount of ^{13}C label incorporated during growth. We estimated the extent of labeling by comparing the signal intensity ratios in labeled and unlabeled ^{13}C spectra, with the isoleucine methyl group as the internal standard. *M. stadtmannae* does not synthesize isoleucine. The ^{13}C abundance of isoleucine is its natural abundance whether or not the cells are grown with enriched precursor.

Materials. All radioisotopes were purchased from New England Nuclear. All gases were analyzed or at least 99.999% pure. All ^{13}C -labeled compounds were 99% enriched (Cambridge Isotope Laboratory, Woburn, Mass.). All other chemicals were of reagent grade quality or better.

RESULTS

Cell carbon precursors. *M. stadtmannae* derived at least 95% of its cell carbon from CO_2 and acetate, with approximately equal contributions by each compound (Table 1). The majority of radioactive carbon from CO_2 and acetate was incorporated into protein (Table 2). Lesser amounts of acetate and CO_2 were incorporated into nucleic acids (Table 2). The amounts incorporated into protein and nucleic acids were similar for both substrates. However, lipids were labeled almost entirely by acetate rather than CO_2 (Table 2). The latter result is expected because the organism cannot synthesize acetate from CO_2 .

M. stadtmannae does not possess a catabolic pathway for reducing CO_2 , via a series of one-carbon (C_1) intermediates, to

TABLE 2. Incorporation of carbon dioxide, acetate, formate, methanol, methionine, and serine into *M. stadtmannae* cell components

^{14}C -labeled precursor	% Radioactivity in:			
	Metabolic intermediates	Lipid	Nucleic acid	Protein
Carbon dioxide	6	3	23	76
U-acetate	2	16	18	64
Formate	4	14	65	18
Methanol	<1	<1	60	39
Methyl, methionine	19	11	28	42
Hydroxymethyl, serine	10	3	38	50

TABLE 3. Amino acid composition of the protein hydrolysate from *M. stadtmanae*

Amino acid	Amt of amino acid (mg/100 mg of cells)	Mol% of amino acid
Glutamate	10.1	16.3
Aspartate	5.8	10.5
Lysine	5.7	9.3
Leucine	4.8	8.6
Isoleucine	4.0	7.3
Alanine	3.4	9.1
Valine	3.4	6.9
Serine	2.7	6.1
Arginine	2.5	3.5
Glycine	2.5	8.0
Threonine	2.5	5.0
Phenylalanine	2.1	3.0
Tyrosine	1.9	2.4
Methionine	1.5	2.5
Histidine	1.1	1.6

methane (17, 18). It is restricted to reducing methanol with H₂. We investigated whether the organism could incorporate possible intermediates of reductive C₁ biosynthetic pathways. The ¹⁴C-labeled C₁ precursors were methanol, formate, methionine methyl, and hydroxymethyl of serine. None of the compounds were significant sources of cell carbon (Table 1). Methanol and formate were preferentially incorporated into nucleic acids (Table 2). In contrast, most of the methionine methyl and serine hydroxymethyl were incorporated into protein (Table 2).

Amino acid composition. The concentrations of 15 amino acids detected in protein hydrolysates are given in Table 3. Proline was present in the hydrolysate but was not quantified owing to the low absorbance of its ninhydrin derivative in the detector. Tryptophan and most of the cysteine present in proteins are destroyed by the acidic hydrolysis. Methionine

and histidine were the least abundant of the amino acids detected in *M. stadtmanae*. Glutamate and aspartate were the most abundant amino acids. However, these amino acids are also the acid hydrolytic products of glutamine and asparagine, respectively. The values in Table 3 do not include the concentrations of glutamate, alanine, lysine, and serine in the pseudomurein cell sacculus of the organism (12). Despite these limitations, the total amount of amino acids detected was 54% of the dry cell weight. Using the Lowry et al. (15) procedure with bovine serum albumin as the standard, we estimated that the total protein content was 61.9% of the dry cell weight.

Incorporation of [¹³C]acetate and ¹³CO₂. The ¹⁴C isotopic experiments showed that almost all cellular carbon was synthesized from acetate and CO₂. We grew the organism with [1-¹³C]acetate, and [2-¹³C]acetate, and ¹³CO₂ to determine the pathways of biosynthesis of amino acids. The chemical shift values for specific amino acid carbons labeled by each precursor are summarized in Table 4. With the exception of isoleucine and leucine, all of the amino acids detected in Table 3 were also found in the ¹³C-labeled hydrolysates. Isoleucine and leucine are required for good growth of the organism, and the amino acids were present in the medium (18). There were no unidentified NMR signals in the spectra.

Multiple labeling and the required carbon carbon coupling were observed for six amino acids grown with ¹³CO₂ as shown in Table 4. For example, the resonances from the β carbon at 25.94 ppm and the γ carbon at 127.25 ppm of histidine are doublets with a coupling constant of 49 Hz. Similar patterns were found for the α and carboxyl carbons of proline, arginine, and glutamic acid. Finally, the *para* and *meta* aromatic carbons of phenylalanine and tyrosine were also coupled.

For the amino acids arginine, proline, and glutamic acid, the same enriched sites were found when *M. stadtmanae* was grown with either methyl-labeled acetate or carboxyl-labeled acetate as precursors. In all cases, these were the β and γ carbons and the extent of labeling was the same starting from either pre-

TABLE 4. Assignments and origins of peaks appearing in the spectra acquired for three hydrolysate samples

Amino acid	Chemical shift (ppm) ^a for:					
	COOH ^b	C-α	C-β	C-γ	C-δ	C-ε
Gly	170.25 ⁺	40.89*				
Ala	173.38 ⁺	49.63*	16.15 ± 0.05°			
Val		59.29*	29.86*	17.90°/18.31°		
Pro	172.25 ^{++c}	60.50 ^{++c}	29.16 ± 0.05*°	24.25 ± 0.01*°	47.09 ⁺	
Ser		55.63*	60.17 ± 0.01°			
Thr		59.39*	66.10°	19.75 ⁺		
Lys	172.58 ⁺	53.62*	27.09°	22.28 ⁺	30.02°	39.87*
Arg	172.32 ^{++c}	53.22 ^{++c}	27.75 ± 0.04*°	24.63 ± 0.01*°	41.16 ⁺	157.60 ⁺
His	171.37°	52.81*	25.94 ^{++d}	127.25 ^{++d}	118.92*	135.10 ± 0.04°
Glu	172.11 ^{++c}	52.95 ^{++c}	25.63 ± 0.07*°	30.35 ± 0.07*°	177.18 ⁺	
Asp	173.82 ⁺	50.21*	34.50°	174.14 ⁺		
Met		52.75*	29.65 ± 0.05°		14.80 ± 0.10°	
Phe	172.46 ⁺	55.24*	36.33°	134.57*	130.32°	130.06°/130.16 ^{++e}
Tyr		55.13*	35.48°	126.55*	131.75°	116.77°/116.78 ^{++e}

^a Chemical shift values were calibrated to the dioxane methylene peak at 67.4 ppm. Symbols: ⁺, CO₂; *, C-1 in acetate; °, C-2 in acetate.

^b The carboxyl group attached to the α-carbon.

^c Average values of coupled doublets. ¹J_{cc} = 59 Hz.

^d Average values of coupled doublets. ¹J_{cc} = 49 Hz.

^e Average values of coupled doublets. ¹J_{cc} = 43 Hz.

p-C128.76^{++e}
p-C155.96^{++e}

FIG. 1. The 75.5-MHz ^{13}C NMR spectra for amino acid carbons. Cells were grown with $[3\text{-}^{13}\text{C}]$ serine (NT = 4,000) (A) and without $[3\text{-}^{13}\text{C}]$ serine (NT = 5,000) (B). Line broadening (1 Hz) was applied to both spectra. (C) The difference spectrum in selected regions is indicated by the dashed lines.

cursor. Additionally, the *meta* carbons of phenylalanine and tyrosine were labeled with either carboxyl-labeled acetate or labeled CO_2 .

Incorporation of $[3\text{-}^{13}\text{C}]$ serine and $[^{13}\text{C}]$ methanol. The ^{14}C isotopic studies showed that only about 1% of the cell carbon was labeled by the hydroxymethyl of serine or by methanol. However, we grew cells with the ^{13}C -enriched precursors to

determine whether specific sites of incorporation into amino acids would reveal special features of the reductive one carbon biosynthetic pathway.

Figure 1 displays and Table 5 summarizes the distribution of ^{13}C label in amino acids of *M. stadtmanae* grown in the presence of hydroxymethyl-labeled serine. The extent of ^{13}C label incorporation is significantly reduced, as expected from the ^{14}C

TABLE 5. Assignments and origins of peaks appearing in the spectra acquired for hydrolysate samples from labelled acetate, serine, and methanol

Amino acid	Chemical shift (ppm) ^a for:					
	COOH ^b	C- α	C- β	C- γ	C- δ	C- ϵ
Ala			16.15 $\pm$ 0.05 ^{°~}			
Val				17.90 [°] /18.31 [°]		
Pro			29.16 $\pm$ 0.05 ^{*°}	24.25 $\pm$ 0.01 ^{*°}		
Ser			60.17 $\pm$ 0.01 ^{°~#}			
Thr			66.10 [°]			
Lys			27.09 [°]		30.02 [°]	
Arg			27.75 $\pm$ 0.04 [°]	24.63 $\pm$ 0.01 [°]		
His	171.37 [°]					135.10 $\pm$ 0.04 ^{°~#}
Glu			25.63 $\pm$ 0.07 [°]	30.35 $\pm$ 0.07 [°]		
Asp			34.50 [°]			
Met			29.65 $\pm$ 0.05 ^{°~}		14.80 $\pm$ 0.10 ^{°~#}	
Phe			36.33 [°]		130.32 [°]	
Tyr			35.48 [°]		131.75 [°]	

^a Chemical shift values were calibrated to the dioxane methylene peak at 67.4 ppm. Symbols: °, C-2 in acetate; ~, C-3 in serine; #, CH_3OH .

^b The carboxyl group attached to the α -carbon.

FIG. 2. The 75.5-MHz ^{13}C NMR spectra for amino acid carbons. Cells were grown with $[^{13}\text{C}]$ methanol (NT = 33,357) (A) and without $[^{13}\text{C}]$ methanol (NT = 32,304) (B). Line broadening (1 Hz) was applied to both spectra. (C) The difference spectrum in selected regions is indicated by the dashed lines.

radioactive data. Nevertheless, some features can be easily discerned. For the hydrolysate derived from enriched serine, Fig. 1A reveals the presence of three new signals (relative to an unlabeled control) in the shielded region and Fig. 1B reveals the presence of one new signal in the deshielded region (relative to an unlabeled control). These are highlighted in the subtracted spectrum in Fig. 1C. The amino acids labeled from the hydroxymethyl of serine were identified to be methionine (δ carbon), alanine (β carbon), and histidine (ϵ carbon), along with β -carbon-enriched serine as expected.

Figure 2 displays and Table 5 summarizes the labeling of amino acid carbons derived from enriched methanol. Figure 2A reveals the presence of three new signals relative to the unlabeled control (Fig. 2B). In the hydrolysate of the protein

fraction with *M. stadtmanae* grown in the presence of ^{13}C -enriched methanol, the signals at 14.8, 60.2, and 135.1 ppm are identified as the δ methyl (C-5) of methionine, β carbon (C-3) of serine, and ϵ carbon (C-7) of histidine.

Table 6 records the extent of enrichment at the various carbon positions, using the assumption and calculations described in the experimental section. The amount of enrichment of particular carbon atoms reinforces the assignments of carbon atoms in Tables 4 and 5. In particular for proline, arginine, glutamic acid, and histidine, for which a double-labeling pattern is demonstrated in the assignments, the estimated enrichments are close to half of the other values, as would be expected. For example, with methyl-labeled acetate, the enrichments of the β and γ carbons of proline are the same and

TABLE 6. Estimated extent of labelling on the relevant sites

Amino acid	Labelling extent ^a						
	COOH	C- α	C- β	C- γ	C- δ	C- ϵ	Other
Gly							
Ala			0.70°/0.04~				
Val				0.60°			
Pro		0.75 ⁺	0.45°	0.45°	0.80 ⁺		
Ser			0.80°				
Thr			0.70°	0.75 ⁺			
Lys			0.70°	0.95 ⁺	0.70°		
Arg		0.95 ⁺	0.35°	0.35°	0.80 ⁺	0.90 ⁺	
His			0.90 ⁺	0.90 ⁺		0.35°/0.20~ / 0.06 [#]	
Glu		0.85 ⁺	0.35°	0.45°	0.80 ⁺		
Asp			0.70°	1.00 ⁺			
Met			0.70°/0.04~		0.80°/0.45~ / 0.25 [#]		
Phe			0.20°		0.25°	0.85 ⁺	p-C0.85 ⁺
Tyr			0.25°		0.25°	0.85 ⁺	

^a Decimal values refer to the molar ratios of putatively labelled molecules over total molecules. Those of α -carboxyl are not listed. The error is ± 0.10 . Symbols: ⁺, CO₂; °, C-2 in acetate; ~, C-3 in serine; #, CH₃OH.

approximately half that expected for a carbon atom uniquely labeled from this precursor.

DISCUSSION

The general labeling patterns of amino acid carbons show that acetate and CO_2 are assimilated into cell carbon of *M. stadtmanae* by pathways common to other members of the methanogenic *Archaea* (2–6, 22, 23, 25). The results denote formation of pyruvate by reductive carboxylation of acetate to pyruvate and incorporation of the pyruvic acid carbon skeleton into alanine, serine, and cysteine. Serine is formed from the pyruvate skeleton by the formation of 3-phosphoglycerate by the reverse of the Embden-Meyerhof-Parnas pathway and conversion of phosphoglycerate to phosphoserine and serine. Glycine is formed by the removal of the β carbon of serine. The labeling of valine is consistent with conventional pathways for converting pyruvate to α -acetolactate and for converting the carbon skeleton of α -acetolactate to the amino acid.

The labeling in aspartate signifies that its carbon skeleton was derived from oxaloacetate formed from pyruvate and carbon dioxide. Threonine and the nonmethyl carbons of methionine were formed from the carbons of aspartate. The methyl carbon of methionine originated from the methyl of acetate. The origins of the carbon skeleton of lysine agree with synthesis from aspartic-semialdehyde by the diaminopimelic acid pathway.

The methyl and carboxyl carbons of acetate were equally distributed between the β and γ carbons of glutamate, arginine, and proline. This indicates that α -ketoglutarate is formed from oxaloacetate via the symmetrical intermediate, succinate, by the reducing reactions of the incomplete tricarboxylic acid pathway. The labeling of the aromatic rings of phenylalanine and tyrosine corresponds to their synthesis from erythrose-4-phosphate via shikimic and chorismic acids.

Histidine labeling is consistent with its formation from 5-phosphoribosyl-1-pyrophosphate (PRPP), ATP, and glutamine. Although this route of synthesis by methanogens was suggested by Ekiel et al. (5), their methods for labeling and NMR analysis were not sensitive enough to define the source of the carbon skeleton of histidine. The definitive results presented in this report are due to greater incorporation of label into cell carbon in *M. stadtmanae*. There is no competition between biosynthetic and catabolic uses of CO_2 and acetate as there is in other methanogens that produce CH_4 from CO_2 and/or acetate. *M. stadtmanae* does not produce CH_4 from either substrate. The β carbon and the γ carbon of histidine are derived from CO_2 , and the δ carbon is derived from the carboxyl group of acetate. It was possible to see both doublets of the required doublet-labeled β carbon and γ carbon. Also in contrast to other studies, a clear signal at 118.9 ppm was obtained for the δ carbon atom when carboxyl-enriched acetate was present in the growth medium.

The labeling patterns of amino acid carbons derived from erythrose-4-phosphate and PRPP imply that the organism synthesizes hexose by the reverse Embden-Meyerhof-Parnas pathway and generates pentose via the oxidative pentose phosphate cycle. Choquet et al. (3) used ^{13}C NMR spectroscopy to study ribose synthesis in six methanogens, including *M. stadtmanae*. The labeling patterns they found in the ribose moiety of purified ribonucleosides from *M. stadtmanae* demonstrated the operation of an oxidative pentose phosphate pathway.

The results of this study show that the methyl group of acetate is clearly the predominant precursor of the hydroxymethyl of serine, the methyl of methionine, and the ϵ carbon (C-7) of histidine (Fig. 3). The labeling of the same carbon

FIG. 3. Biosynthetic C₁ transfer reactions in *M. stadtmanae*.

positions by serine hydroxymethyl confirmed that serine is the direct donor of C₁ units for biosynthetic reactions in *M. stadtmanae*. Glycine is formed when the hydroxymethyl carbon of serine is transferred as a methylene-C₁ to an unknown C₁ carrier compound. The methylene-C₁ is reduced to a methyl derivative or oxidized to the methenyl derivative and hydrated to formyl-C₁. Choquet et al. (2) provided evidence that a formyl C₁ unit is the direct precursor of the C-2 position of the purine ring synthesized by *M. stadtmanae*. The C-2 position of the purine moiety of ATP is incorporated directly into the ϵ position of histidine.

Labeled alanine was formed from labeled serine without the intermediate formation of pyruvate. No label was detected in any of the other amino acids formed from pyruvate or serine, including aspartate, which is present in large amounts in the protein hydrolysates examined. Since the pyridoxal phosphate enzyme serine dehydrase is ubiquitous and alanine labeling from acetate and CO_2 is consistent with formation from pyruvate, the result was unexpected. We hypothesize that a minor pathway from serine to alanine is present. This could involve the elimination of water from the serine and reduction to yield alanine.

The relative contribution of methanol carbon to C₁ biosynthetic reactions is much less than that of acetate methyl carbon. Only ca. 1% of the total cell carbon is derived from methanol. However, the ^{13}C studies clearly show that methanol can contribute methyl-C₁ units for methionine biosynthesis. Much less methanol carbon was oxidized to the formyl level and incorporated into the C-7 position of histidine. This suggests that the methyl-C₁ units from methanol are used more rapidly in methyl transfer reactions than they are oxidized to the methenyl-C₁ level. Methanol also labeled the hydroxymethyl of serine. A serine transhydroxymethylase reaction has been described in *Methanobacterium thermoautotrophicum* (8). The reaction is usually reversible in other organisms.

The chemical nature of the C₁-carrier cofactor in *M. stadtmanae* is not known. Typical derivatives of tetrahydromethanopterin (H₄MPT) are not found at detectable levels in extracts (11, 24). Low levels of methylene-H₄MPT dehydrogenase and methylene-H₄MPT reductase activities are found in extracts of *M. stadtmanae* (21, 24). Both activities were tested by the addition of methylene-H₄MPT to extracts. The enzyme activities may reflect reactions involved in biosynthetic C₁ transfer

reactions; however, it is premature to speculate that H₄MPT is the physiological C₁ carrier in *M. stadtmanae*.

Solution NMR techniques indicate that pyruvate is formed by *M. stadtmanae* by reductive carboxylation of acetate. The carbon skeletons of serine, alanine, and cysteine arise directly from pyruvate, and other amino acids are formed from pyruvate and CO₂ by conventional biosynthetic pathways. We present the first demonstration for a methanogen of the synthesis of the methyl group of methionine from the methyl group of acetate. This means that the β carbon of serine is the precursor of the methyl group, and this conclusion is reinforced and confirmed by the enrichment of the methionine methyl by labeled methanol and β-labeled serine. We also present unambiguous proof that the C-7 of histidine arises from the methyl group of acetate, and we show the origin of the other carbons of the amino acid.

ACKNOWLEDGMENTS

E. Currenti, E. Kusel, and M. Nicpon provided technical assistance. We thank Liming ChangChien, Wadsworth Center, for determining the amino acid composition. We also thank R. Weinbloom and M. J. Tenney, Wadsworth Center, for carbon analysis.

This study was supported in part by grant DCB90-17471 from the National Science Foundation.

REFERENCES

- Chen, M., and M. J. Wolin. 1977. Influence of methane production by *Methanobacterium ruminantium* on the fermentation of *Selenomonas ruminantium*. *Appl. Environ. Microbiol.* **34**:756–759.
- Choquet, C. G., J. R. Richards, G. B. Patel, and G. D. Sprott. 1994. Purine and pyrimidine biosynthesis in methanogenic bacteria. *Arch. Microbiol.* **161**:471–480.
- Choquet, C. G., J. R. Richards, G. B. Patel, and G. D. Sprott. 1994. Ribose biosynthesis in methanogenic bacteria. *Arch. Microbiol.* **161**:481–488.
- Ekiel, I. A., K. F. Jarrell, and G. D. Sprott. 1985. Amino acid biosynthesis and sodium-dependent transport in *Methanococcus voltae*, as revealed by ¹³C NMR. *Eur. J. Biochem.* **149**:437–444.
- Ekiel, I. A., I. C. P. Smith, and G. D. Sprott. 1983. Biosynthetic pathways in *Methanospirillum hungatei* as determined by ¹³C nuclear magnetic resonance. *J. Bacteriol.* **156**:316–326.
- Ekiel, I. A., G. D. Sprott, and G. B. Patel. 1985. Acetate and CO₂ assimilation by *Methanotherx concilii*. *J. Bacteriol.* **162**:905–908.
- Fuchs, G., and E. Stupperich. 1980. Autotrophic CO₂ fixation pathway in *Chlorobium limicola*. Evidence for the operation of a reductive tricarboxylic acid cycle in growing cells. *Arch. Microbiol.* **128**:64–71.
- Hoyt, J. C., A. Oren, J. C. Escalante-Semerena, and R. S. Wolfe. 1986. Tetrahydromethanopterin-dependent serine transhydroxymethylase. *Arch. Microbiol.* **145**:153–158.
- Jones, W. J., M. I. Donnelly, and R. S. Wolfe. 1985. Evidence of a common pathway of carbon dioxide reduction to methane in methanogens. *J. Bacteriol.* **163**:126–131.
- Jones, W. J., D. P. Nagle, Jr., and W. B. Whitman. 1987. Methanogens and the diversity of archaeobacteria. *Microbiol. Rev.* **51**:135–177.
- Keltjens, J. T., and G. D. Vogels. 1988. Methanopterin and methanogenic bacteria. *Biofactors* **1**:95–103.
- König, H. 1986. Chemical composition of cell envelopes of methanogenic bacteria isolated from human and animal feces. *Syst. Appl. Microbiol.* **8**:159–162.
- Krieg, N. R., and P. Gerhardt. 1981. Solid culture, p. 143–150. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
- Lajoie, S. F., S. Bank, T. L. Miller, and M. J. Wolin. 1988. Acetate production from hydrogen and [¹³C]carbon dioxide by the microflora of human feces. *Appl. Environ. Microbiol.* **54**:2723–2727.
- Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
- Miller, T. L., and M. J. Wolin. 1974. A serum bottle modification of the Hungate technique for culturing obligate anaerobes. *Appl. Microbiol.* **27**:985–987.
- Miller, T. L., and M. J. Wolin. 1983. Oxidation of hydrogen and reduction of methane is the sole energy source for a methanogen isolated from human feces. *J. Bacteriol.* **153**:1051–1055.
- Miller, T. L., and M. J. Wolin. 1985. *Methanospaera stadtmaniae* gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. *Arch. Microbiol.* **141**:116–122.
- Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1963. Studies of biosynthesis in *Escherichia coli*. Carnegie Institution Washington Publication 607, Washington, D.C.
- Rouviere, P. E., and R. S. Wolfe. 1988. Novel biochemistry of methanogenesis. *J. Biol. Chem.* **263**:7913–7916.
- Schwörer, B., and R. K. Thauer. 1991. Activities of formylmethanofuran dehydrogenase, methylenetetrahydromethanopterin dehydrogenase, methylenetetrahydromethanopterin reductase, and heterodisulfide reductase in methanogenic bacteria. *Arch. Microbiol.* **155**:459–465.
- Simpson, P. G., and W. B. Whitman. 1993. Anabolic pathways in methanogens, p. 445–489. In J. G. Ferry (ed.), *Methanogenesis*. Chapman & Hall, New York.
- Sprott, G. D., I. Ekiel, and G. B. Patel. 1993. Metabolic pathways in *Methanococcus jannaschii* and other methanogenic bacteria. *Appl. Environ. Microbiol.* **59**:1092–1098.
- Van der Wijngaard, W. M. H., J. Creemers, G. D. Vogels, and C. Van der Drift. 1991. Methanogenic pathways in *Methanospaera stadtmanae*. *FEMS Microbiol. Lett.* **80**:207–212.
- Whitman, W. B. 1994. Autotrophic acetyl coenzyme A biosynthesis in methanogens, p. 521–538. In H. L. Drake (ed.), *Acetogenesis*. Chapman & Hall, New York.