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Autotrophic Acetyl Coenzyme A Biosynthesis in *Methanococcus maripaludis*

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To detect autotrophic CO_2 assimilation in cell extracts of *Methanococcus maripaludis*, lactate dehydrogenase and NADH were added to convert pyruvate formed from autotrophically synthesized acetyl coenzyme A to lactate. The lactate produced was determined spectrophotometrically. When CO_2 fixation was pulled in the direction of lactate synthesis, CO_2 reduction to methane was inhibited. Bromoethanesulfonate (BES), a potent inhibitor of methanogenesis, enhanced lactate synthesis, and methyl coenzyme M inhibited it in the absence of BES. Lactate synthesis was dependent on CO_2 and H_2, but H_2 + CO_2-independent synthesis was also observed. In cell extracts, the rate of lactate synthesis was about 1.2 nmol min^{-1} mg of $protein^{-1}$. When BES was added, the rate of lactate synthesis increased to 2.3 nmol min^{-1} mg of $protein^{-1}$. Because acetyl coenzyme A did not stimulate lactate synthesis, pyruvate synthase may have been the limiting activity in these assays. Radiolabel from $^{14}CO_2$ was incorporated into lactate. The percentages of radiolabel in the C-1, C-2, and C-3 positions of lactate were 73, 33, and 11%, respectively. Both carbon monoxide and formaldehyde stimulated lactate synthesis. $^{14}CH_2O$ was specifically incorporated into the C-3 of lactate, and ^{14}CO was incorporated into the C-1 and C-2 positions. Low concentrations of cyanide also inhibited autotrophic growth, CO dehydrogenase activity, and autotrophic lactate synthesis. These observations are in agreement with the acetogenic pathway of autotrophic CO_2 assimilation.

Most species of methanogenic bacteria can oxidize hydrogen and reduce carbon dioxide to methane to obtain energy for growth (26, 47). In addition, about one-half of the species of methanogens which have been described are able to grow autotrophically. Nevertheless, the pathway of CO_2 fixation during autotrophic growth in methanogens has not been fully established, and the carbon metabolism of only a few species has been characterized. In *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*, a novel pathway of acetyl coenzyme A (acetyl-CoA) synthesis from two molecules of CO_2 has been proposed (16, 21, 29, 42–44). This pathway closely resembles the total synthesis of acetate described previously in *Clostridium thermoaceticum* and other anaerobic eubacteria (8, 13, 22, 24, 25, 32, 33, 50).

The acetyl-CoA pathway in *C. thermoaceticum* involves total synthesis of acetate from two molecules of CO_2 via different routes of reduction (32, 50). One molecule of CO_2 is reduced to the methyl level via the folate pathway, and the other molecule of CO_2 is reduced to a bound CO residue. They are then condensed to an activated acetyl residue which is thiolized with HS-CoA. The final step of acetyl-CoA biosynthesis is catalyzed by CO dehydrogenase (36).

In *Methanobacterium thermoautotrophicum*, acetyl-CoA is an early product of CO_2 assimilation (15, 17, 38). Labeling studies have shown that the biosynthesis of acetyl-CoA in *Methanobacterium thermoautotrophicum* resembles the total synthesis of acetyl-CoA from $2CO_2$ by acetogens. The methyl group of acetate comes from methyltetrahydromethanopterin (methyl-THMPT), which is a folate analog and an intermediate in the reduction of CO_2 to methane (11, 28, 30, 45). The carboxy group of acetyl-CoA is derived from another molecule of CO_2, which is reduced to a bound CO by CO dehydrogenase, or it can come directly from carbon monoxide gas (46). Thus, autotrophic CO_2 assimilation and CO_2 reduction to methane have intermediates in common (21).

In this report, we describe a spectrophotometric assay for the acetogenic pathway in cell extracts of *Methanococcus maripaludis*. Using this assay, we also demonstrated the presence of this pathway for the first time in the *Methanococcales*, one of three major orders of methane-producing bacteria (26, 51).

MATERIALS AND METHODS

Bacteria and growth conditions. *Methanococcus maripaludis* JJ was grown anaerobically on H_2-CO_2 (80:20 [vol/vol]) gas at 37°C in mineral medium as described previously (27, 48). A typical fermentor was prepared as follows. Ten liters of mineral medium was autoclaved under N_2 gas for 40 min, and 20 ml of a sterile solution containing 10 g of 2-mercaptoethanesulfonate and 5 ml of a sterile solution of 20% (wt/vol) $Na_2S \cdot 9H_2O$ was then added. The fermentor was inoculated with 1 liter of culture. Sterile 20% $Na_2S \cdot 9H_2O$ (5 ml) was added to the fermentor twice a day during growth. The pressure of H_2 + CO_2 gas was maintained at 100 kPa, and the flow rate was 100 to 250 ml/min. Cells were harvested in the early stationary phase with a Sharples continuous-flow centrifuge and stored under H_2 gas at −20°C.

Preparation of extracts. Cell pellets were thawed in an anaerobic chamber (Coy Laboratory, Ann Arbor, Mich.). Cells were lysed by addition of 25 mM potassium-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, pH 6.8, containing 1 mM dithiothreitol and 1 mM cysteine, which was saturated with H_2 gas. Pancreatic DNase, 1 mg/20 g (wet weight) of cells, was added. The cell extract became homogeneous after 30 min of incubation in the chamber at room temperature, and it was then centrifuged at 30,000 × *g* for 30 min at 4°C in a polypropylene centrifugation bottle which had been equilibrated in an anaerobic chamber for at least 1 day. The supernatant was collected in an anaerobic chamber

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and was either used immediately or stored under H_2 gas at −20°C. Under these conditions, activity was retained for 6 months. The protein contents of extracts were determined by the method of Lowry et al. (34) after digestion in 0.1 M NaOH for 30 min at 90°C. Similar results were obtained by the dye-binding method (3).

In vitro lactate synthesis. Assays were performed in 2.6-ml serum vials with butyl rubber stoppers and aluminum seals (West Co., Inc., Phoenixville, Pa.). Before the assays, extracts were preincubated under H_2 gas to remove endogenous substrates. Typically, 4 ml of extract was incubated for 1 h at 37°C in a 160-ml serum bottle under an atmosphere of H_2 at 200 kPa. Lactate dehydrogenase (LDH) and other components of the assay were prepared in 50 mM Tricine [*N*-Tris(hydroxymethyl)methyl glycine] buffer (pH 7.5; Sigma Chemical Co., St. Louis, Mo.). Except for LDH, oxygen-stable components were dispensed in small volumes (usually 10 μl) into the assay vials outside the anaerobic chamber. During passage through the air lock into the chamber, the solutions were maintained in vacuo for 5 min during one of the three normal cycles. The vials were then sealed inside the chamber. Stoppers for the vials were taken into the chamber at least 2 days before use. LDH was prepared separately in a total volume of 0.4 ml in a serum vial. The vial was stoppered, and the stopper was punctured with a 22-gauge needle. In this way, the gas in the vial was exchanged with N_2 gas during passage through the air lock to the anaerobic chamber. Upon removal from the anaerobic chamber, the assay vials were placed in an ice bath and flushed with H_2 gas for 5 min at a flow rate of 10 ml/min. Flushing with H_2 continued during addition of extracts and LDH. Cell extract (0.2 ml containing 8 mg of protein) was dispensed into the assay vials with a 1-ml Glas-pak disposable syringe (Becton Dickinson Vacutainer Systems, Rutherford, N.J.). After addition of the extracts, LDH (10 μl containing 25 U) and bromoethanesulfonate (BES; 10 μl containing 20 nmol) were added to the vials with a 50 μl syringe (Hamilton Co., Reno, Nev.). Upon addition of the last component, the vials were flushed with H_2-CO_2 (80:20 [vol/vol]) for 5 min. The assays were initiated by transfer of the vials to a 37°C water bath. After 40 min, the vials were transferred to an ice bath and a 20-μl gas sample was removed from each vial with a 100-μl gas-tight syringe (Precision Sampling Corp., Baton Rouge, La.) and analyzed for methane by gas chromatography (49). The extracts were then acidified by addition of 0.2 ml of 60% $HClO_4$, transferred to 1.5-ml centrifuge tubes, and centrifuged for 4 min at 10,000 × *g* at room temperature. After neutralization of 0.2 ml of the supernatant with 0.1 ml of 1.5 M K_2CO_3, lactate was measured spectrophotometrically with LDH and NAD^+ (20).

Pyruvate synthase was measured by a similar procedure. The only difference was that 20 μmol of acetyl phosphate and 5 U of phosphotransacetylase were included in the assays under H_2-CO_2.

All assays were performed in duplicate, and the results shown are the averages. In addition, results were reproduced at least once. In most cases, extracts from more than one batch of cells were tested. When cyanide was used, the pH of the assay was increased by 0.4 U with 20 mM KCN.

Incorporation of radiolabel into lactate. In radiolabeling experiments, $^{14}CO_2$, ^{14}CO, and $^{14}CH_2O$ were added to the assays after removal of the gassing needles. The specific activity of the $^{14}CO_2$ was determined from a sample of the gas phase. The quantity of CO_2 was determined by gas chromatography with a thermal conductivity detector (Varian 3760; Sugarland, Tex.) under the same conditions used to analyze CH_4. Radioactivity was measured by liquid scintillation counting after trapping of the radiolabel in CO_2-trapping cocktail. ^{14}CO was prepared from radiolabel formate (14). The specific activity of ^{14}CO was calculated from the specific activity of the labeled formate, assuming complete conversion. The specific activities of $^{14}CO_2$, ^{14}CO, and $^{14}CH_2O$ used in these experiments were 6.64×10^6, 1.36×10^6, and 6.83×10^6 dpm/μmol, respectively. At the conclusion of the radiolabeling experiment, extracts were acidified as described above. After centrifugation, a portion of the supernatant was assayed for lactate. Unlabeled lactate (1 mmol) was added to the remaining supernatant as a carrier.

Purification and degradation of lactate. Lactate was purified by chromatography on Celite (Johns-Manville Products Corp., Manville, N.J.) and Dowex 50W, H^+ (1, 18). The radiochemical purity of lactate was confirmed by thin-layer chromatography on silica gel plates with chloroform–1-butanol (80:20) and fluorography (2). On KB silica gel plates (Whatman Inc., Clifton, N.J.), the R_f of lactate was 0.14. Before the degradation, an additional 1 mmol of unlabeled lactate was added. C-1 degradation of lactate was performed by dichromate oxidation (1). Acetate, which is the product, was purified from the lysate by steam distillation, taken to dryness, suspended in about 1 ml of distilled water, and degraded further by the Schmidt method (1). Total degradation of lactate to $3CO_2$ was performed by the Van Slyke-Folch oxidation procedure (39). The $^{14}CO_2$ released from each degradation step was absorbed in a 2 N NaOH solution whose weight had been carefully predetermined. The amount of CO_2 trapped in the NaOH solution was then calculated from the increase in weight. The amount of radiolabel trapped was determined by liquid scintillation counting.

Formation of $^{14}CO_2$ from radiolabeled substrates. The rate of formation of $^{14}CO_2$ from $^{14}CH_2O$ and ^{14}CO was determined under the conditions of the standard assay, except that the volume was increased. In a 10-ml serum bottle, 0.5 ml of extract (18 mg of protein) was incubated with 6 μmol of NADH and 60 U of LDH under an atmosphere of H_2-CO_2 (80:20 [vol/vol]). Radiolabeled $^{14}CH_2O$ (2.5 μmol containing 5.6×10^6 dpm) or ^{14}CO (0.4 mmol containing 5.1×10^6 dpm was then added. After 40 min at 37°C, the gas inside the bottle was flushed out by a stream of N_2 gas into CO_2-trapping cocktail, and the radioactivity was determined by liquid scintillation counting.

Enzymatic assays. CO dehydrogenase was assayed in 1 ml of 50 mM phosphate buffer, pH 7.5, which contained 20 mM methyl viologen, at 25°C. The cuvettes were stoppered with red serum rubber stoppers and made anaerobic by repeated vacuuming and flushing with N_2 gas. The cuvettes were then flushed with 100% CO gas for 30 s and pressurized to 100 kPa. The CO gas was made oxygen free by being flushed through a solution of 25 mM methyl viologen, which was reduced with about 10 mM sodium dithionite. Enough sodium dithionite was then added to the cuvettes to turn the reaction mixture slightly blue. The assay was initiated by injection of the cell extract. The reduction of methyl viologen was monitored at 603 nm with a Gilford spectrophotometer. The extinction coefficient of methyl viologen was 11.3×10^3 cm^{-1} M^{-1}. The activity was linear with protein from 0.2 to 0.8 mg. Pyruvate oxidoreductase was assayed in 100 mM potassium-Tricine buffer, pH 8.6, which contained 20 mM methyl viologen. The cuvettes were prepared in the manner used for the CO dehydrogenase assay except that

TABLE 1. LDH trap for autotrophic acetyl-CoA synthesis

Addition(s) (amt)	ΔA_{340}[a]	Lactate formed (nmol)	CH_4 formed (nmol)
None[b]	0.052	0	2,800
NADH (2.5 μmol)	0.031	0	3,700
LDH (25 U)	0.060	11	3,100
NADH + LDH	0.330	388	1,400

[a] Change in A_{340} observed in the spectrophotometric assay for lactate.
[b] With no additions, the assay included 8 mg of protein under an atmosphere of H_2 + CO_2.

the gas phase was N_2. After addition of cell extract, the reaction was initiated by addition of sodium pyruvate (final concentration, 10 mM) and CoA (final concentration, 0.1 mM). Reduction of methyl viologen was again monitored at 603 nm. The reaction was both pyruvate- and CoA dependent, and it was linear with protein from 0.2 to 0.8 mg.

Materials. All chemicals were reagent grade or better. H_2 and H_2-CO_2 (80:20 [vol/vol]) were purchased from Selox Co. (Gainesville, Ga.). Carbon monoxide was obtained from Fisher Scientific (Springfield, N.J.). Rabbit muscle lactate dehydrogenase (type XI), bovine pancreatic DNase (type IV), NAD^+, NADH, acetyl-CoA, acetyl phosphate, ATP, ADP, AMP, pyruvate, lactate, PIPES, and Tricine buffers were obtained from Sigma. BES was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). $NaH^{14}CO_3$, $^{14}CH_2O$ (30 mCi/mmol), and [^{14}C]formate (40 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, Calif.). Methyl coenzyme M (methyl-CoM) was prepared as described previously (49). $^{14}CO_2$ gas was prepared from $NaH^{14}CO_3$. A solution containing 8.4 mg of $NaH^{14}CO_3$ (5 mCi/mmol) in 5 ml of sterile water was injected with a syringe into a 10-ml serum bottle containing N_2 gas at 50 kPa. One milliliter of 6 M HCl was then added with a syringe, and the solutions were gently mixed. The gas phase was transferred with a short length of tubing to a second serum bottle which had previously been evacuated. The first bottle was then filled with a saturated solution of NaCl to effect complete transfer of the gas phase to the second bottle. After disconnection of the tubing between the two bottles, 2 ml of 2.5% (wt/vol) $Na_2S \cdot 9H_2O$ was added to the bottle containing the $^{14}CO_2$. The bottle was then incubated on a rotary shaker for 24 h to eliminate contamination by O_2. CO_2-trapping cocktail contained 0.5 liter of toluene, 0.4 liter of methanol, 0.1 liter of ethanolamine, and 2 g of 2,5-bis[5′-*tert*-butylbenzoxazolyl-(2′)]thiophene.

RESULTS

Spectrophotometric assay for acetyl-CoA synthesis. In cell extracts, acetyl-CoA synthesis was barely detectable, presumably because acetyl-CoA was rapidly metabolized to other compounds. Therefore, an assay was devised to trap pyruvate formed from autotrophically synthesized acetyl-CoA and pyruvate synthase as lactate by addition of LDH and NADH. Extracts of *Methanococcus maripaludis* synthesized lactate when supplemented with LDH and NADH under an H_2-CO_2 atmosphere (Table 1). A small change in absorbance was also observed during the spectrophotometric determination of lactate in extracts incubated without these components. This change was also observed when LDH was omitted from the subsequent spectrophotometric assay for lactate. Therefore, interference by chromophores in the extract limited the sensitivity of the assay. Although these chromophores could be removed by dialysis, lactate synthesis activity was also greatly reduced (data not shown). Under the standard assay conditions, lactate synthesis was linear with the amount of extract added, and exogenous pyruvate was recovered almost quantitatively (data not shown). The temperature optimum for lactate synthesis was 37°C or close to the growth temperature of *Methanococcus maripaludis*. The loss in activity above 37°C was not due to inactivation of the LDH, which retained activity up to 55°C. These results established the conditions for lactate synthesis in extracts of *Methanococcus maripaludis*.

TABLE 2. Effect of gas atmosphere during the assay and preincubation on lactate synthesis and methanogenesis

Atmosphere of assay	Atmosphere of preincubation[a]	Lactate synthesis (nmol)[b]	CH_4 formation (nmol)[c]
H_2-CO_2	H_2	239	2,580
N_2-CO_2	H_2	167	310
H_2	H_2	176	390
N_2	H_2	149	290
H_2-CO_2	N_2	145	1,450
N_2-CO_2	N_2	151	60
H_2	N_2	150	230

[a] Extracts were preincubated under H_2 or N_2 for 60 min.
[b] The assay included 2.5 mg of protein, 2.5 μmol of NADH, 25 U of LDH, and 0.1 mM BES. The assay lasted 40 min.
[c] CH_4 formation without LDH, NADH, and BES.

Lactate synthesis was dependent on the gas atmosphere of the assay and the preincubation. Lactate synthesis in crude extracts was sensitive to O_2. Subsequent to exposure of the extracts to air for 5 min, lactate synthesis was not observed (data not shown). This result was consistent with the extreme oxygen sensitivity of the enzymes of acetyl-CoA and pyruvate synthesis (52). Extracts were normally preincubated for 1 h before the assay to reduce the endogenous substrates for lactate synthesis. After preincubations under N_2, lactate synthesis and methane production when assayed under H_2-CO_2 were reduced to about 60% of the activity after preincubation with H_2 (Table 2). Presumably, preincubation under H_2 was necessary to conserve the reducing ability of the extracts. The amounts of lactate formed when the assays were performed under N_2-CO_2 or H_2 was reduced to about 70% of the activity under H_2-CO_2 (Table 2). Thus, lactate synthesis was partially dependent on both H_2 and CO_2. Because lactate synthesis was observed in the absence of CO_2, endogenous substrates were probably a source of some of the lactate.

In extracts of *Methanobacterium thermoautotrophicum*, methyl-CoM stimulates acetyl-CoA synthesis from CO_2, presumably because it stimulates CO_2 reduction to methyl-THMPT and CH_4 (19, 30, 37, 44). Likewise, the inhibitor of the methyl-CoM reductase system, BES, inhibits acetyl-CoA synthesis because it prevents reduction of CO_2 (44). In extracts of *Methanococcus maripaludis*, neither NADH nor LDH alone inhibited methanogenesis from H_2-CO_2, but when they were combined methanogenesis was inhibited by 50% (Table 1). This observation supported the hypothesis that lactate synthesis competes for an intermediate of methanogenesis. However, in extracts of *Methanococcus maripaludis*, methyl-CoM inhibited lactate synthesis, and BES stimulated lactate synthesis (Table 3). Therefore, the rate of lactate synthesis was probably not limited by the availability of C-1 units from methanogenesis. Alternatively, both lactate synthesis and methanogenesis required reducing equivalents from H_2. Even though H_2 was present in great excess, electron transfer may have been disrupted during prepara-

TABLE 3. Effects of methyl-CoM and BES on lactate synthesis

Addition(s) (amt)	Lactate formed (nmol)	CH_4 formed (nmol)
None[a]	420	1,300
BES (0.1 mM)	600	200
Methyl-CoM (1 μmol)	330	2,100
BES + methyl-CoM	700	200

[a] With no additions, the assay included 8 mg of protein, 2.5 μmol of NADH, 25 U of LDH, and H_2-CO_2 gas.

tion of the extracts. Therefore, the availability of a common reductant may have been limiting.

Lactate synthesis was linear with time for at least 1 h (Fig. 1). In the presence or absence of BES, the specific activity of lactate synthesis was 2.3 or 1.2 nmol min^{-1} mg of $protein^{-1}$, respectively. The CO_2-dependent lactate synthesis was also proportional to the CO_2 concentration in the assay (Fig. 2). The concentration of CO_2 for 50% activity was about 2%. When acetyl-CoA was generated in the assay, the concentration of CO_2 required for 50% activity of pyruvate synthase was 1%. At CO_2 concentrations higher than 5%, the maximal specific rate of CO_2-dependent pyruvate synthesis in this assay was about 1.2 nmol min^{-1} mg of $protein^{-1}$. This shows

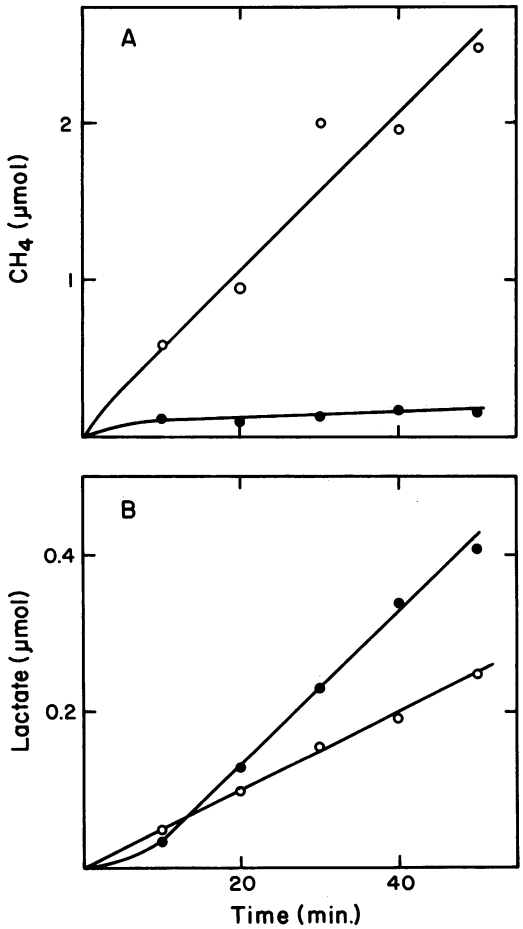

FIG. 1. Rate of lactate and methane synthesis from H_2-CO_2. The assays were performed under standard conditions in the presence (●) or absence of (○) of 0.1 mM BES.

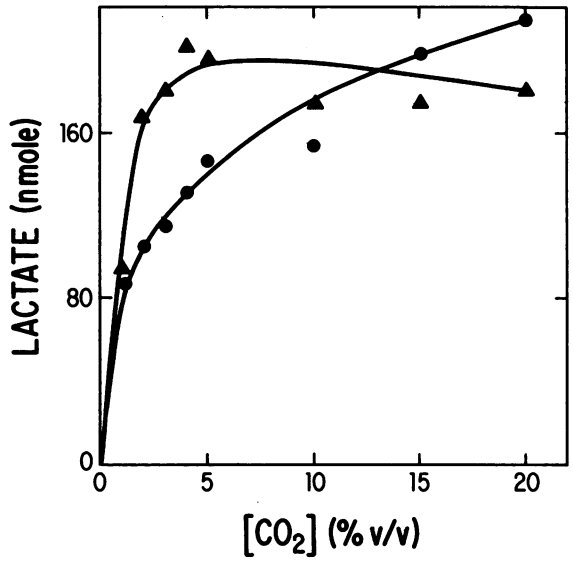

FIG. 2. Effects of CO_2 and acetyl-CoA on lactate synthesis. Extracts, 4 mg of protein, were assayed under standard conditions as described in the text. Symbols: ●, without acetyl-CoA; ▲, with acetyl-CoA-generating system. CO_2-independent lactate synthesis was subtracted.

that the maximal specific rate of CO_2-dependent acetyl-CoA formation in this assay is ≥1.2 nmol min^{-1} mg of $protein^{-1}$.

Radiolabel from $^{14}CO_2$ was incorporated into lactate (Table 4). Most of the radiolabel was incorporated into the C-1 position. This result would be expected if pyruvate was formed from acetyl-CoA and CO_2 by pyruvate synthase (40). Somewhat less radiolabel, 45 and 15% of the amount in the C-1 position, was found in the C-2 and C-3 positions of lactate, respectively (Table 4). The uneven labeling of the C-2 and C-3 positions could have resulted from acetyl-CoA synthesis from endogenous C-1 units and CO_2 or an exchange reaction of CO_2 and the C-1 of acetyl-CoA (C-2 of lactate) (36).

Pathway of CO_2 assimilation. In other methanogens, THMPT and CO are C-1 donors for autotrophic acetyl-CoA biosynthesis (30, 31). CH_2O condenses with THMPT to form methylene-THMPT in extracts, and it is an intermediate in methanogenesis or incorporated into the methyl group of acetyl-CoA (12, 31). CO is incorporated into the carboxy group. In extracts of *Methanococcus maripaludis*, CH_2O was converted quantitatively to methane (data not shown). Thus, CH_2O appeared to have the same mode of action as found in other methanogens (12). In the presence of BES added to inhibit methanogenesis, CH_2O, and CO stimulated

TABLE 4. Degradation of radiolabeled lactate formed from $^{14}CO_2$

Carbon atom(s) of lactate	Specific radioactivity of CO_2 (10^3 dpm/mmol)	% of radiolabel in lactate[a]
All	24.7	
C-1	54.0	73
C-2	24.1	33
C-3	8.0	11

[a] The total (100%) radiolabel in lactate was equal to three times the specific activity of the CO_2 obtained by the total degradation of lactate. Thus, the enrichment of each carbon was obtained from the relative specific radioactivity of the CO_2 released during degradation.

TABLE 5. Stimulation of lactate synthesis by CH_2O and CO

Addition(s) (amt or pressure)	Lacate formed (nmol)	CH_4 formed (nmol)
None[a]	510	100
CH_2O (1 μmol)	520	90
CO (100 kPa)	620	60
CH_2O + CO	730	90

[a] With no additions, the assay included 8 mg of protein, 2.5 μmol of NADH, 25 U of LDH, 0.1 mM BES, and H_2-CO_2 gas.

lactate synthesis (Table 5). Although the stimulation was only about 40%, it was very reproducible and was obtained with extracts from at least five batches of cells. $^{14}CH_2O$ and ^{14}CO specifically radiolabeled the C-3 and C-2 carbons of lactate, respectively (Table 6). For $^{14}CH_2O$, 71% of the total radiolabel was incorporated into the C-3 carbon of lactate, which corresponded to the methyl carbon of acetyl-CoA. To determine whether incorporation of radiolabel into carbons C-1 and C-2 was due to oxidation of $^{14}CH_2O$ to $^{14}CO_2$, the rate of this reaction was measured. Under the conditions of the lactate synthesis assay, extracts converted $^{14}CH_2O$ to $^{14}CO_2$ at a rate of 0.87 nmol min^{-1} mg of $protein^{-1}$. Thus, about one-third of the initial $^{14}CH_2O$ was converted to $^{14}CO_2$ by the end of the assay. For ^{14}CO, only small amounts of radiolabel were incorporated. However, the C-2 of lactate, which corresponded to the carboxy of acetyl-CoA, was specifically enriched (Table 6). Most of the remaining radiolabel was found in the C-1 of lactate, which was probably derived from CO_2 (Table 4). Like CH_2O, ^{14}CO was converted to $^{14}CO_2$ by these extracts. Under the conditions of the lactate synthesis assay, ^{14}CO was converted to $^{14}CO_2$ at a rate of 0.2 nmol min^{-1} mg of $protein^{-1}$.

In acetogenic bacteria, the immediate methyl donor for acetyl-CoA synthesis is a corrinoid protein (7, 23). In *Methanobacterium thermoautotrophicum*, methyl iodide is a methyl donor, presumably because it chemically methylates a corrinoid intermediate (21). In extracts of *Methanococcus maripaludis*, methyl iodide also stimulated lactate synthesis in the presence of CO (Table 7). The stimulation was reproducible, and it was observed in extracts from two batches of cells. This observation suggested that a corrinoid protein is an intermediate in acetyl-CoA synthesis in *Methanococcus maripaludis* as well.

The pathway of autotrophic CO_2 assimilation in *Methanococcus maripaludis* appeared to resemble closely that found in other methanogens and the acetogenic eubacteria. This conclusion was further confirmed by detection of high specific activities of enzymes usually associated with this pathway (Table 8). The CO dehydrogenase activity, assayed in the direction of methyl viologen reduction, was 4.3 μmol of methyl viologen reduced min^{-1} mg of $protein^{-1}$. CO dehydrogenase was also a major protein in extracts of the closely related bacterium *Methanococcus vannielii* (5). Likewise, the methyl viologen-dependent pyruvate oxidoreductase activity was 70 nmol of methyl viologen reduced min^{-1} mg of $protein^{-1}$. These levels of activity were comparable to those previously reported for these enzymes in methanogens. It is noteworthy that the oxidative capabilities of both of these enzymes far exceeded the biosynthetic or physiological activities, which were determined from the rate of lactate synthesis. Thus, the level of lactate biosynthetic activity in these extracts was generally 2 to 3 nmol of lactate synthesized min^{-1} mg of $protein^{-1}$ in the presence of BES. In the absence of BES, the activity was somewhat lower (Table 8). After growth with acetate, extracts of *Methanococcus maripaludis* contained lower levels of CO dehydrogenase, pyruvate oxidoreductase, and lactate synthesis activity than did autotrophically grown cells (Table 8). These results would be expected if the synthesis of the enzymes for autotrophic CO_2 fixation were repressed during heterotrophic growth.

TABLE 7. Stimulation of lactate synthesis by methyl iodide and CO

Addition(s) (amt or pressure)	Lactate formed (nmol)	CH_4 formed (nmol)
None[a]	480	180
CH_3I (5 mM)	540	10
CO (100 kPa)	540	20
CH_3I + CO	650	20

[a] With no addition, the assay contained 7 mg of protein, 2.5 μmol of NADH, 25 U of LDH, 0.1 mM BES, and H_2-CO_2 gas.

Inhibition by cyanide. CO dehydrogenase is sensitive to low concentrations of cyanide, and inhibition by cyanide is diagnostic of pathways which use this enzyme in many anaerobes (4, 6, 9, 10, 35, 41). A low concentration of cyanide, 0.2 mM, abolished autotrophic growth of *Methanococcus maripaludis*, and acetate partially restored growth (data not shown). Likewise, the concentration of cyanide required for 50% inhibition (I_{50}) of methyl viologen-dependent CO dehydrogenase activity was 0.3 mM. Much higher concentrations of cyanide were required to inhibit autotrophic acetyl-CoA biosynthesis measured by the lactate trap (Fig. 3). In the presence of 20% CO_2, the I_{50} was near 20 mM. Because this concentration of cyanide also inhibited lactate synthesis in the presence of an acetyl-CoA-generating system, inhibition may have been due to formation of the cyanohydrin of pyruvate or another indirect effect.

TABLE 6. Degradation of radiolabeled lactate formed from $^{14}CH_2O$ and ^{14}CO

Carbon atom(s) of lactate	Specific radioactivity (10^3 dpm/mmol) of CO_2 (% of radiolabel) with:	
	$^{14}CH_2O$	^{14}CO
All	9.4	0.50
C-1	4.0 (14)	0.71 (47)
C-2	2.3 (8)	0.76 (51)
C-3	20.1 (71)	0.19 (13)

[a] The total (100%) radiolabel in lactate was equal to three times the specific activity of the CO_2 obtained from the total degradation of lactate (see Table 4 footnote).

TABLE 8. Enzymes associated with autotrophy in *Methanococcus maripaludis*

Enzymatic activity	Sp act (nmol min^{-1} mg of $protein^{-1}$) after growth[a]	
	Without acetate	With acetate
Acetyl-CoA synthase[b]	1.25	0.42
CO dehydrogenase (EC 1.2.99.2)[c]	4,300	95
Pyruvate oxidoreductase (EC 1.2.7.1)[c]	66	28

[a] The results are averages of at least three determinations.
[b] Rate of lactate synthesis in the absence of BES.
[c] Methyl viologen dependent.

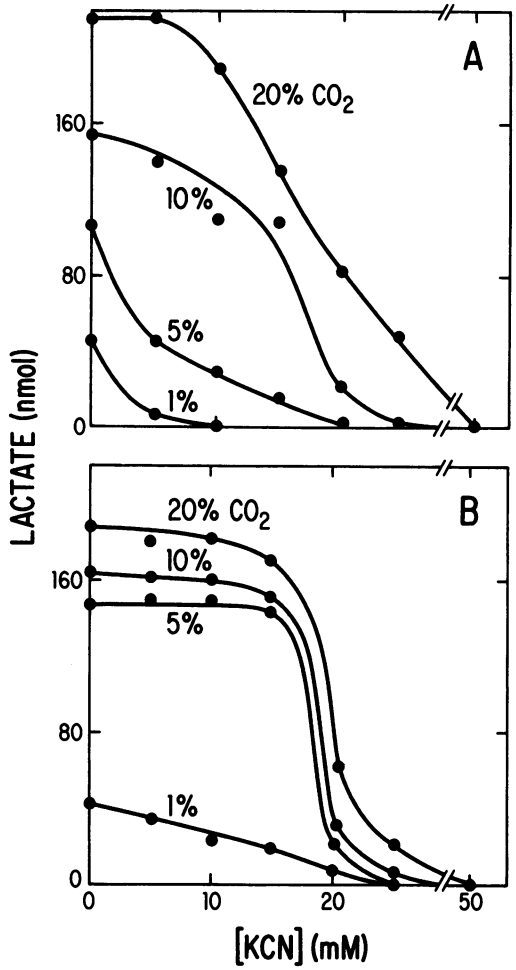

FIG. 3. Inhibition of autotrophic acetyl-CoA synthesis and pyruvate synthase by cyanide. (A) Extracts (4 mg of protein) were assayed for autotrophic acetyl-CoA synthesis and pyruvate synthase with an LDH trap for pyruvate. (B) Extracts (3 mg of protein) were assayed for pyruvate synthase alone in the presence of an acetyl-CoA-generating system. The CO_2 atmosphere of the assay is given in percent.

However, in the presence of 1 or 5% CO_2, the I_{50} for acetyl-CoA biosynthesis was reduced to about 2 or 5 mM, respectively (Fig. 3). At these concentrations, cyanide did not inhibit lactate synthesis in the presence of the acetyl-CoA-generating system. Therefore, inhibition may be attributed to a direct effect on acetyl-CoA synthesis. The requirement for high concentrations of cyanide for inhibition may be due to protection by CO_2 in the assay and disruption of the enzyme system upon preparation of the extracts.

Cyanide also inhibited pyruvate synthesis at low concentrations of CO_2. Thus, at 1% CO_2 the I_{50} was 10 mM (Fig. 3B). Higher concentrations of CO_2 were protective. Likewise, in the methyl viologen-dependent assay, the I_{50} for the pyruvate oxidoreductase was 2 mM (data not shown).

DISCUSSION

The evidence that the autotrophic acetyl-CoA biosynthetic pathway is operative in *Methanococcus maripaludis* is as follows. (i) Cell extracts prepared from autotrophically grown cells contain high levels of CO dehydrogenase activity. This activity is essential for autotrophic CO_2 fixation via this pathway. Moreover, CO dehydrogenase activity is greatly reduced in extracts of heterotrophically grown cells. (ii) Lactate synthesis from CO_2 is stimulated by formaldehyde, methyl iodide, and carbon monoxide. These compounds are C-1 donors for autotrophic acetyl-CoA biosynthesis (21, 31, 46). (iii) The pattern of $^{14}CO_2$ incorporation into lactate is consistent with the proposal that lactate was formed in part by autotrophic acetyl-CoA biosynthesis. Moreover, $^{14}CH_2O$ and ^{14}CO specifically radiolabel the C-3 and C-2 carbons of lactate, respectively. (iv) Autotrophic growth and lactate synthesis are inhibited by cyanide, which is a specific inhibitor of CO dehydrogenase at low concentrations (4, 35). These results further confirm the presence of the autotrophic acetyl-CoA biosynthetic pathway in *Methanococcus maripaludis*.

However, a great deal of uncertainty remains concerning the nature of this pathway in methanococci, as well as in other methanogens. For the most part, arguments for the pathway in methanogens depend heavily upon analogies with the elegant work on *C. thermoaceticum* by Ljungdahl and Wood et al. (32, 50). For instance, the role of a corrinoid protein in methyl transfer has never been demonstrated directly in methanogens. Moreover, fundamental differences between the methanogenic and clostridial system may exist, especially in the activation of CO_2. In clostridia, CO_2 is activated by formate dehydrogenase and formyl-tetrahydrofolate synthetase. ATP is required for this latter enzyme (32). In methanogens, formyl-THMPT is synthesized without a stoichiometric requirement for ATP (M. I. Donnelly and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-56, p. 174). Instead, activation of CO_2 appears to be coupled to the methylreductase system (19, 37). Therefore, the pathway of autotrophic acetyl-CoA biosynthesis, which removes intermediates of methanogenesis for cell carbon synthesis, must also restore C-1 intermediates if methanogenesis is to continue. The nature of these anaplerotic reactions is not known.

Lactate synthesis in these extracts underestimates the true rate of autotrophic CO_2 assimilation by 1 or 2 orders of magnitude. Assuming a cellular protein content of 70% and that 60% of the cell carbon is derived from acetate (40), the rate of acetate synthesis necessary to support the specific growth rate of 0.27 h^{-1} during autotrophic growth is 80 nmol min^{-1} mg of $protein^{-1}$. However, considering the complexity of these reactions and the sensitivity of these catalysts to minute quantities of oxygen, the low in vitro activity was not unexpected. Low rates of acetyl-CoA biosynthesis from H_2 and CO_2 (0.1 to 0.2 nmol min^{-1} mg of $protein^{-1}$) were also observed in Triton X-100-treated cell suspensions, as well as in cell extracts of *Methanobacterium thermoautotrophicum* (44). Moreover, even in an ammonium sulfate fraction of an extract of *Methanobacterium thermoautotrophicum*, the rates of acetyl-CoA biosynthesis were only 15 nmol min^{-1} mg of extract $protein^{-1}$ (31).

Methanogens are a very diverse group of archaebacteria (51). Previous studies of carbon fixation in methanogens have focused on *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*, which represent two of the three major phylogenetic groups of methanogens. In this paper, we report on carbon metabolism in *Methanococcus maripaludis*, which represents the third phylogenetic group of methanogens. We have established a simple spectrophotometric assay to monitor the initial CO_2 assimilation in extracts of *Methanococcus maripaludis*. By labeling and enzyme studies, we have demonstrated the presence of the

autotrophic acetyl-CoA pathway in this bacterium. Therefore, the acetyl-CoA biosynthetic pathway for autotrophic CO_2 fixation is present in representatives of each major phylogenetic group of methanogens.

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