STIMULATION OF CO_2 REDUCTION TO METHANE BY METHYL-COENZYME M IN EXTRACTS OF METHANOBACTERIUM

Robert P. Gunsalus and R. S. Wolfe

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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SUMMARY Addition of methyl-coenzyme M ($CH_3SCH_2CH_2SO_3^-$) to undialized, anaerobic, cell-extracts of Methanobacterium thermoautotrophicum under an atmosphere of H_2 and CO_2 (80:20 v/v) stimulates 30-fold the rate of CO_2 reduction to methane. For each mol of $CH_3SCH_2CH_2SO_3^-$ added 12 mol of methane is produced. This stimulation phenomenon requires magnesium ion, ATP, H_2, and $CH_3SCH_2CH_2SO_3^-$. Neither the reduced form of the cofactor, $HSCH_2CH_2SO_3^-$, nor the oxidized, disulfide form will replace the methylated coenzyme.

Results of biochemical studies have demonstrated that coenzyme M acts as a methyl transfer coenzyme in the terminal steps of methane formation (1, 2, 3). In the system studied by Taylor the reduced form of the coenzyme, HS-CoM*, accepts the methyl group from exogenous methylcobalamin to yield CH_3-S-CoM; this reaction is catalyzed by methylcobalamin-CoM-methyltransferase (4). CH_3-S-CoM is then reductively demethylated to methane and HS-CoM, with H_2 as the source of reducing potential, by the Mg and ATP-dependent methylcoenzyme M reductase system. McBride has demonstrated that cell extract, when pulsed with [^{14}C] CO_2, results in the generation of [^{14}C-methyl] CH_3-S-CoM. These data demonstrated that CH_3-S-CoM is an intermediate in the reductive pathway from CO_2 to methane; it is unclear how the more oxidized one-carbon intermediate steps proceed.

*Abbreviations: CH_3-S-CoM, methylcoenzyme M or 2-(methylthio) ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid; $(S\text{-CoM})_2$, 2,2'-dithiodiethanesulfonic acid; $(CH_3)_2$-$\overset{+}{S}$-CoM, 2-(dimethylsulfonium) ethanesulfonate; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

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Here we describe a system in which CH_3-S-CoM stimulates the reduction of CO_2 to CH_4 in cell extracts of the thermophilic organism, Methanobacterium thermoautotrophicum. We have found that the total CO_2 reduced to CH_4 is proportional to the amount of CH_3-S-CoM added and that the reaction requires the addition of magnesium ion and ATP in addition to CH_3-S-CoM. Other forms of the coenzyme: HS-CoM, $(S\text{-CoM})_2$, and $(CH_3)_2\overset{+}{S}$-CoM do not replace CH_3-S-CoM in the stimulation phenomenon.

MATERIALS AND METHODS

Cells of M. thermoautotrophicum were grown in a salts medium at 60° as previously described (5). Carbon and energy were supplied to the cultures by constant sparging with a H_2 and CO_2 gas mixture (80:20 v/v) at a rate of 200 cc per minute. Cells were harvested by continuous Sharples centrifugation after 36 h of growth (late log phase). Harvested cells were diluted with an equal volume of 50 mM TES buffer, pH 7.0, and were gassed vigorously with H_2 via the Hungate technique to remove oxygen (6). The resulting cell slurry was broken by passage through a French pressure cell at 12,000 psi and collected under a gentle stream of H_2 in stainless steel centrifuge tubes (2.8 x 10 cm). The broken cell suspension was centrifuged for 30 min at 33,000 x g under a H_2 atmosphere, the resulting supernant solution was decanted into tubes and regassed with H_2. This extract was then stored at -20° until used.

Methane production was assayed with small serum stoppered reaction vials as described by Taylor (2). The standard reaction mixture (0.25 ml) contained: 30 μmol TES buffer, pH 6.0 (when measured at the assay temperature of 60°); 5 μmol Mg Cl_2; 1 μmol ATP: CH_3-S-CoM, cell extract and gas phase as indicated. The reaction was initiated by transfer of the reaction vials to a shaking water bath at 60°. Gas aliquots (20 μl) were withdrawn by Hamilton syringe and injected into a Packard Model 300 gas chromatograph for methane analysis (2). CH_3-S-CoM, HS-CoM, $(CH_3)_2\overset{+}{S}$-CoM, and $(S\text{-CoM})_2$ were synthesized as described by Taylor (2). ATP and TES buffer were purchased from Sigma Chemical Co.; H_2, N_2 and CO_2 gasses were purchased from Linde Co. Trace amounts of O_2 were removed from the gasses by passage through a glass column which contained copper filings at 350°.

RESULTS

CH_3-S-CoM is a potent stimulator of CO_2 reduction to methane in cell extracts of M. thermoautotrophicum (Fig. 1). When cell extract was incubated in the presence of CO_2 (20% gas phase) and CH_3-S-CoM (200 nmol),

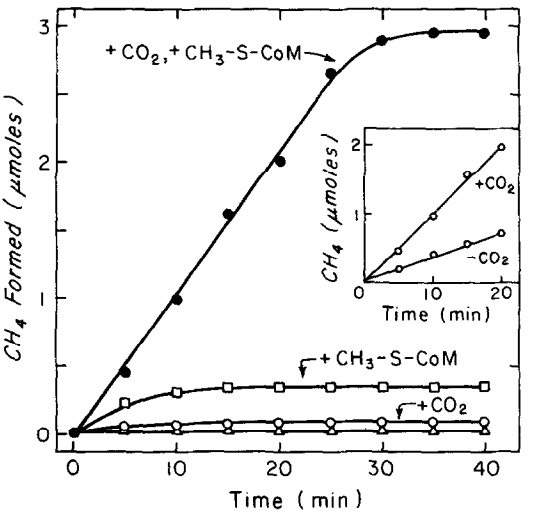

Fig. 1 Stimulation of CO_2 reduction by CH_3-S-CoM: Effect of reaction components on CH_4 formation. Each reaction vial contained the standard reaction components described in Materials and Methods plus 50 μl cell extract (1.7 mg protein) under a H_2 atmosphere. Additions to the indicated vials were: 0.2 μmol CH_3-S-CoM (□—□); 20% CO_2 gas phase, balance H_2 (○—○); 0.2 μmol CH_3-S-CoM plus 20% CO_2 gas phase (●—●); none (Δ—Δ). Insert shows reaction time course in presence and absence of CO_2 gas phase and 2 μmol CH_3-S-CoM.

2900 nmol of CH_4 were formed over a period of 30 min. Under identical conditions in the absence of CH_3-S-CoM, only about 100 nmol of CH_4 was formed. When CO_2 was omitted from the reaction mixture, slightly more methane was formed (300 nmol) than would be expected from the 200 nmol CH_3-S-CoM precursor added. These small differences may be due to traces of dissolved CO_2 and/or other C-1 precursors present in the cell extract. In the absence of CO_2 and CH_3-S-CoM, no methane was detected.

The initial rate of methane formation may vary significantly; from CO_2 alone the rate of methane formation is roughly 0.087 μmol formed per hr per mg protein. When CH_3-S-CoM is added, the rate increases to 2.67 μmol per hr per mg protein. Thus, the overall rate of methane formation is stimulated about 30-fold by CH_3-S-CoM. Increasing the concentration

Table 1: Requirements for CH_3-S-CoM stimulated CO_2 reduction to CH_4.

Reaction conditions omissions[a]	CH_4 formed (nmoles per 30 minutes)
none	2,705
-Mg	24
-ATP	0
-CH_3-S-CoM	178
-cell extract	0
-H_2	3
-CO_2	306

[a]Each reaction vial received where indicated: 30 μmol TES buffer, pH 6.0; 5 μmol $MgCl_2$; 1 μmol ATP; 0.2 μmol CH_3-S-CoM; 50 μl cell extract, 2.7 mg protein in a reaction volume of 0.25 ml. The reaction time was 30 min at 60°. The gas phase was H_2:CO_2 mixture (80:20 v/v) except when CO_2 was omitted (balance H_2) or H_2 omitted (balance N_2).

of CH_3-S-CoM in the reaction mixture 10 fold (Insert Fig. 1) has no additional effect on the rate of stimulated methane formation; however, this rate of formation is 2.8-fold over the optimal rate of methane formation from CH_3-S-CoM in the absence of CO_2.

The requirements for CH_3-S-CoM-stimulated CO_2 reduction to methane are shown in Table 1. Magnesium ion and ATP requirements are similar to those observed for the methyl-coenzyme M reductase reaction (1). Whether these two components are required for earlier steps of CO_2 reduction to CH_3-S-CoM is unclear. Omission of H_2 from the reaction vial results in no methane generation as reducing equivalents are not available for the reductive steps from CO_2 to CH_4. When CH_3-S-CoM is replaced by other forms of the coenzyme, HS-CoM, $(S\text{-CoM})_2$, or $(CH_3)_2\overset{+}{S}$-CoM, stimulation is not observed. $(CH_3)_2\overset{+}{S}$-CoM does not serve as a substrate for methane formation in extracts of M. thermoautotrophicum.

As shown in Fig. 2, methane production was dependent solely on the addition of CH_3-S-CoM to the reaction mixture. Under a H_2 atmosphere, methane was formed in stoichometric amounts after each addition of

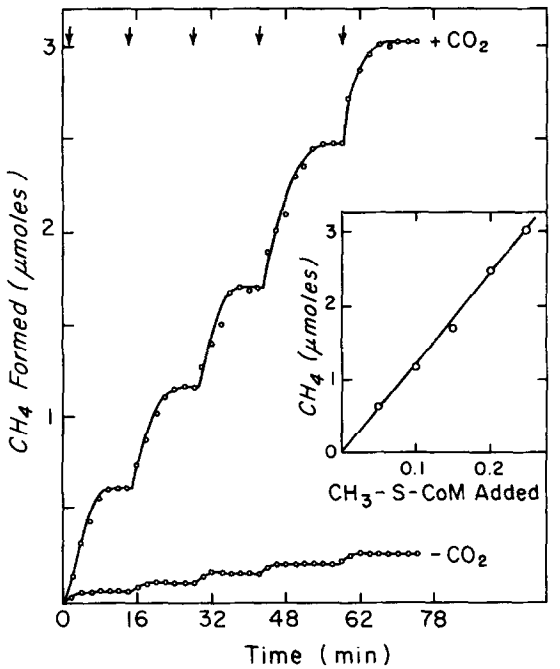

Fig. 2 Effect of CH_3-S-CoM addition on the amount of methane formed. Reaction components and conditions were as described in Table 1 except that CH_3-S-CoM (50 nmol) was added at the times indicated by the arrows. Insert shows the total amount of methane produced after each addition plotted versus the total amount of CH_3-S-CoM added.

CH_3-S-CoM (50 nmol CH_3-S-CoM was added at each time period indicated by an arrow). In the presence of CO_2, methane production occurred in the stimulated fashion after each CH_3-S-CoM addition and the process could be reinitiated a number of times. When the total amount of methane produced after each addition is plotted versus the total amount of CH_3-S-CoM added, a straight line relationship is observed (Inset Fig. 2). The ratio of mol CH_4 formed per mol CH_3-S-CoM added is 12. Eleven molecules of CO_2 are fully reduced to CH_4 per 1 reduced from CH_3-S-CoM. It was not necessary to add additional ATP to the reaction mixture as the system remained fully active during the time course of the experiment. Although the role of ATP in the demethylation reaction of CH_3-S-CoM is unclear, ATP is only required in catalytic amounts.

DISCUSSION

It is interesting that the requirements for the stimulation phenomenon are similar to those for the methylcoenzyme M reductase reaction. At present it cannot be determined if early steps of CO_2 reduction also require Mg:ATP. The fact that neither HS-CoM nor $(S\text{-}CoM)_2$ replace $CH_3\text{-}S\text{-}CoM$ in stimulation suggest that an intermediate generated in the $CH_3\text{-}S\text{-}CoM$ methylreductase reaction may be responsible for the activation and subsequent reduction of CO_2 to methane. The dimethylsulfonium analog of CoM which does not serve as a substrate for methane will not stimulate CO_2 reduction either. Barker (7) has proposed that a hypothetical C-1 carrier, X, could be responsible for the activation of CO_2 and subsequent mediation of the following reductive steps. More recently it has been proposed that CoM could act as this carrier (8). The present data would not contradict this possibility. Why the final methylcoenzyme M reduction reaction goes at a reduced rate in the absence of CO_2 is unclear. However, these results show that $CH_3\text{-}S\text{-}CoM$ in addition to serving as a direct precursor of methane, also acts to stimulate CO_2 reduction by an as yet undetermined mechanism at rates approaching methane formation in unbroken whole cells.

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