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Coupling of Methyl Coenzyme M Reduction with Carbon Dioxide Activation in Extracts of *Methanobacterium thermoautotrophicum*

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The stimulation of carbon dioxide reduction to methane by addition of 2-(methylthio)ethanesulfonate (CH_3-S-CoM) to cell extracts of *Methanobacterium thermoautotrophicum* was investigated. Similar stimulation of CO_2 reduction by CH_3-S-CoM was found for cell extracts of *Methanobacterium bryantii* and *Methanospirillum hungatei*. The CH_3-S-CoM requirement could be met by the methanogenic precursors formaldehyde, serine, or pyruvate, or by 2-(ethylthio)ethanesulfonate (CH_3CH_2-S-CoM), but not by other coenzyme M derivatives. Efficient reduction of CO_2 to CH_4 was favored by low concentrations of CH_3-S-CoM and high concentrations of CO_2. Sulfhydryl compounds were identified as effective inhibitors of CO_2 reduction. Both an allosteric model and a free-radical model for the mechanism of CO_2 activation and reduction are discussed.

Methanogens share in common the ability to oxidize hydrogen and reduce carbon dioxide to methane. Formate, formaldehyde, and methanol do not appear to be intermediates in CO_2 reduction (T. C. Stadtman, Ph.D. thesis, University of California, Berkeley, 1949; 13). Barker (1) proposed that the C_1 intermediates in methanogenesis are bound to one or more carriers. Evidence corroborating at least a portion of Barker's reductive reaction pathway was first provided by McBride and Wolfe (6) when they reported the partial purification of a heat-stable, acidic, dialyzable cofactor, CoM, which mediates C_1 transfer from [*methyl*-^{14}C]methylcobalamin ($^{14}CH_3$-B_{12}) or from $^{14}CO_2$ to form $^{14}CH_4$ in cell extracts of *Methanobacterium bryantii*. Subsequently, Taylor and Wolfe (14) identified the active methyl acceptor as HS-CoM and the methylated form of the coenzyme was CH_3-S-CoM. CoM is presently believed to be the terminal C_1 carrier in methanogenesis. Evidence also has been presented suggesting that CoM is the methanogenic C_1 carrier at the formaldehyde level of oxidation (9). The C_1 carrier at the formyl and carboxyl levels of oxidation may be methanopterin, a new factor found in methanogens and characterized as a pterin in the laboratory of Keltjens and Vogels (4).

Recently, Gunsalus and Wolfe (3) showed that reduction of CH_3-S-CoM was coupled to the reduction of CO_2 in extracts of *Methanobacterium thermoautotrophicum*. They found that the rate of CO_2 reduction to CH_4 was stimulated 30-fold by the addition of CH_3-S-CoM and that 11 mol of CO_2 was reduced for each mole of CH_3-S-CoM reduced. Addition of HS-CoM, $(S\text{-}CoM)_2$, and $(CH_3)_2$-S^+-CoM did not replace the requirement for CH_3-S-CoM. Here we further characterize the nature of the coupling between CH_3-S-CoM reduction and CO_2 activation in cell extracts of *M. thermoautotrophicum*. We refer to this phenomenon as the RPG effect (3).

MATERIALS AND METHODS

Abbreviations. CoM, coenzyme M; HS-CoM, 2-mercaptoethanesulfonic acid; CH_3-S-CoM, methyl coenzyme M or 2-(methylthio)ethanesulfonic acid; $(S\text{-}CoM)_2$, 2,2′-dithiodiethanesulfonic acid; CH_3CH_2-S-CoM, 2-(ethylthio)ethanesulfonic acid; $CH_3CH_2CH_2$-S-CoM, 2-(propylthio)ethanesulfonic acid; $(CH_3)_2$-S^+-CoM, 2-(dimethylsulfonium)ethanesulfonic acid; $S{=}(CoM)_2$, thiodiethanesulfonic acid; CHO-S-CoM, 2-(formylthio)ethanesulfonic acid; CH_3CO-S-CoM, 2-(acetylthio)ethanesulfonic acid; $HOOCCH_2$-S-CoM, 2-(carboxymethylthio)ethanesulfonic acid; adenosyl-S-CoM, *S*-(5′-deoxyadenosyl)mercaptoethanesulfonic acid.

M. thermoautotrophicum was grown anaerobically on 80% H_2:20% CO_2 at 60°C, and cell extracts were prepared by the procedures reported by Romesser and Wolfe (9). Assays for methane formation were performed in calibrated 5-ml reaction vials, and gas was measured chromatographically as described initially by Taylor and Wolfe (14) and modified by Romesser and Wolfe (9). A typical assay mixture for methane production contained 75 μmol of PIPES buffer (1,4-piperazinediethanesulfonic acid) at pH 6.6 and 60°C, 1.0 μmol of ATP, 1.0 μmol of $MgCl_2$, 0.5 μmol of CH_3-

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S-CoM, and 100 μl of cell extract in a total volume of 350 to 400 μl. The gas atmosphere was 80% H_2 and 20% CO_2, and each reaction was performed at 60°C. The amount of CO_2 reduced to CH_4 was determined by subtracting the micromoles of CH_4 expected to be formed by complete reduction of added CH_3-S-CoM from the total micromoles of CH_4 measured.

$(S\text{-}CoM)_2$, and CH_3-S-CoM were prepared as described by Romesser and Balch (8). CH_3CH_2-S-CoM, $CH_3CH_2CH_2$-S-CoM, and $S{=}(CoM)_2$ were synthesized as described by Gunsalus et al. (2). $(CH_3)_2$-S^+-CoM was prepared as described by Taylor and Wolfe (14).

CHO-S-CoM was prepared by a modification of the procedure described by Yamada et al. (16). HS-CoM (500 mg, 3.14 mmol) was mixed with 4.7 ml of *N,N'*-dimethylformamide, 119 μl of 98% HCOOH (3.4 mmol), and 1.73 g of diphenylphosphorazidate (6.28 mmol; Aldrich Chemical Co.); the mixture was cooled in an ice bath, and 439 μl of triethylamine (3.14 mmol) was added. The mixture was stirred at 4°C for 3 days and then was poured into 630 ml of acetone. The mixture was stored at −20°C overnight; the precipitate which formed was filtered off and discarded. The filtrate was flash evaporated to an oil; 30 ml of acetone was added, and then ether was added to cause formation of a precipitate. The mixture was stored at −20°C overnight, and the acetone-ether solution was decanted from the clear gummy residue which formed. The liquid was flash evaporated to an oil; the oil was taken up in 15 ml of acetone, and ether was added to separate an oil. The gummy residue and oil were combined in 5 ml of water and applied to a Sephadex SP-C25 (Ca^{2+}) column (2.5 by 20 cm) equilibrated with water. Fractions which gave a single spot when chromatographed on Eastman 6064 cellulose plates in acetone-water (16:3) and sprayed with silver fluoresceinate (12) were pooled and flash evaporated to dryness. CHO-S-CoM (145 mg, 23%) was crystallized from methanol-acetone. Analysis: calculated for $C_6H_{10}O_8S_4Ca \cdot H_2O$: C, 18.2; H, 3.1; S, 32.3; Ca, 10.1. Found: C, 18.1; H, 3.0; S, 32.6; Ca, 10.1.

To prepare CH_3CO-S-CoM, 101 mg of HS-CoM (0.634 mmol) was dissolved in 0.8 ml of formic acid in an ice bath; acetic anhydride (2 ml) was added, and the mixture was incubated for 24 h at 4°C. The reaction mixture was then flash evaporated to dryness, and CH_3CO-S-CoM (119 mg, 93%) was crystallized from methanol-diethyl ether. Analysis: calculated for $C_4H_{11}NO_4S_2$: C, 23.9; H, 5.5; N, 7.0; S, 31.9. Found: C, 24.0: H, 5.5; N, 6.5; S, 31.5.

$HOOCCH_2$-S-CoM (376 mg, 51%) was prepared by alkylation of HS-CoM (500 mg, 3.13 mmol) with ICH_2COOH (585 mg, 3.12 mmol) in 5 ml of NH_4OH. The NH_4OH was made anaerobic by bubbling with N_2 (10 ml/min for 20 min). The reaction was allowed to proceed overnight at 4°C in the dark. The reaction was stopped by pouring the mixture into 50 ml of acetone. The calcium salt was prepared by passing the oil down a Sephadex SP-C25 (Ca^{2+}) column (2.0 by 35 cm) equilibrated with water. The effluent from the column was flash evaporated to dryness, and $HOOCCH_2$-S-CoM was crystallized from aqueous acetone. Analysis: calculated for $C_4H_6O_5S_2Ca$: C, 20.2; H, 2.5; S, 29.9; Ca, 16.8. Found: C, 20.2; H, 2.8; S, 30.0; Ca, 16.2.

Adenosyl-S-CoM was synthesized by a modification of the procedure described by Wang and Hogenkamp (15). HS-CoM (1.11 g, 6.98 mmol) was dissolved in 12 ml of 2 N NaOH, and 1.0 g of 5'-chloro-5'-deoxyadenosine (3.5 mmol; gift of H. P. C. Hogenkamp) was added; the mixture was stirred vigorously at 80°C for 2 h. The mixture was cooled and acidified to pH 6.0 with dilute acetic acid and then was applied to a Dowex 50-X2 (H^+, 2 by 30 cm) column equilibrated with water. The column was first washed with 150 ml of water, and then adenosyl-S-CoM was eluted with 1 M NH_4OH. The absorbance at 260 nm was measured, and each fraction with an absorbance >2 was pooled. The pooled fractions were flash evaporated to dryness, and then adenosyl-S-CoM (562 mg, 39%) was crystallized from hot water. Methanol was added to complete crystallization and to wash the crystals. Analysis: calculated for $C_{12}H_{17}N_5O_6S_2 \cdot H_2O$: C, 35.3; H, 4.4; N, 17.2; S, 15.7. Found: C, 35.7; H, 4.5; N, 17.3; S, 15.7.

The ^1H nuclear magnetic resonance spectra were recorded on a Varian HA-100 spectrometer and were in each case consistent with the assigned structure.

RESULTS

Optimal reaction conditions. Cell extracts of *M. thermoautotrophicum* reduced CO_2 to CH_4 at a rate of 39 nmol h^{-1} mg of $protein^{-1}$. The rate was increased to 1,084 nmol h^{-1} mg^{-1} by the addition of 0.5 μmol of CH_3-S-CoM; approximately 4 mol of CO_2 were reduced per mol of CH_3-S-CoM added. This stimulation of CO_2 reduction (RPG effect) could be repeated many times by further additions of CH_3-S-CoM. These results are consistent with those reported by Gunsalus and Wolfe (3), except that they obtained reduction of 11 mol of CO_2 per mol of CH_3-S-CoM added. We have found the molar yield of CO_2 reduced per CH_3-S-CoM added to vary from 2 to 24 depending on the cell extract preparation and the reaction conditions used.

Reaction conditions for the RPG effect with cell extracts of *M. thermoautotrophicum* were optimized to give maximal conversions of CO_2 to CH_4 (Fig. 1). Maximal CO_2 reduction was observed at pH 6.2 and at buffer concentrations of 200 to 340 mM PIPES. Addition of less than 100 μl of cell extract resulted in decreased CO_2 reduction. As reported previously (3), the RPG effect required ATP. Under the conditions used in these assays, the addition of 0.15 to 1.0 μmol of ATP gave maximal activity. The ATP requirement was catalytic; 17 mol of CO_2 was reduced per mol of ATP added. Mg^{2+} was stimulatory, with optimal activity at an $MgCl_2$ to ATP ratio of 1:1. Inhibition of activity was observed as this ratio exceeded 10:1.

Distribution of the RPG effect in methanogens. Cell extracts of several methanogens were examined for the ability to display an RPG effect. Extracts of *M. bryantii* and *Methanospirillum hungatei* showed an increased rate of methanogenesis and an increase in the amount of CO_2

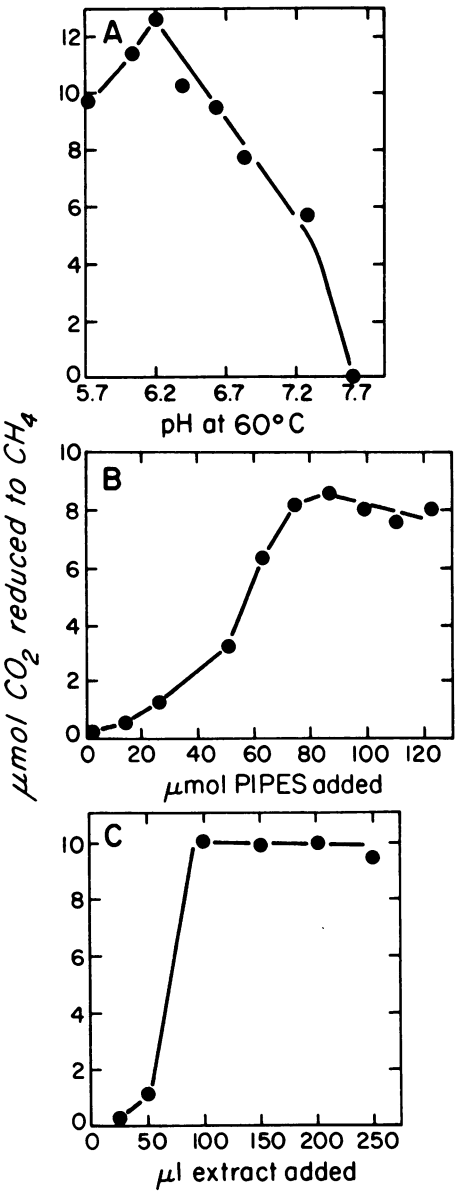

FIG. 1. Optimal reaction conditions for the RPG effect. Formation of CH_4 was measured as described in the text. Each reaction mixture contained (unless otherwise indicated): 48 μmol of PIPES buffer (pH 6.6) at 60°C, 2.5 μmol of $MgCl_2$, 1.25 μmol of ATP, 0.5 μmol of CH_3-S-CoM, and 100 μl of cell extract. The gas atmosphere was 80% H_2 and 20% CO_2. The reaction volume was 350 μl. (A) Effect of pH. (B) Effect of PIPES buffer concentration. (C) Effect of protein concentration (100 μl of extract = 4.5 mg of protein).

reduced to CH_4 when CH_3-S-CoM was added to the reaction mixture in the presence of an H_2-CO_2 atmosphere (Table 1). With extracts of *Methanobacterium formicicum*, *Methanosarcina barkeri*, and *Methanobrevibacter ruminantium*, on the other hand, little or no evidence of an RPG effect was found.

Induction of the RPG effect. When added to cell extract in the presence of an H_2 atmosphere, not only CH_3-S-CoM but also HCHO, serine, and sodium pyruvate provided precursors that were reduced to methane (Table 2); CH_3CH_2-S-CoM was reduced to C_2H_6. In the presence of an H_2 and CO_2 atmosphere, these same compounds markedly stimulated the amount of CO_2 reduced to CH_4. Several other CoM derivatives were tested for their activity in cell extracts and for their ability to stimulate CO_2 reduction. The thiolesters CHO-S-CoM and CH_3CO-S-CoM were found to be enzymically hydrolyzed at 980 and 225 nmol h^{-1} mg of protein^{-1}, respectively. Adenosyl-S-CoM, $S=(CoM)_2$, $CH_3CH_2CH_2$-S-CoM, and $HOOCCH_2$-S-CoM were not metabolized; none of these CoM derivatives caused an RPG effect when added to cell extracts, and it was shown previously (3) that HS-CoM (S-$CoM)_2$, and $(CH_3)_2$-S^+-CoM did not replace CH_3-S-CoM in eliciting the RPG effect. Additional compounds tested as methanogenic precursors and as inducers of the RPG effect which were inactive in both tests included calcium lactate, methanol, formate, acetate, homoserine, serinol, isoserine, and α-methylserine.

It was found (Table 2) that methanogenic precursors at the methyl (CH_3-S-CoM), hydroxymethyl (serine), and carboxyl (pyruvate) levels of oxidation were each able to induce the RPG effect. These results suggest that the stage of CO_2 reduction stimulated in the RPG effect is one preceding the point at which the carboxyl group of compounds such as pyruvate enter the methanogenic pathway.

Effect of CH_3-S-CoM concentration. Gunsalus and Wolfe (3) found that when sequential additions of CH_3-S-CoM were made to cell extracts of *M. thermoautotrophicum*, the amount of CO_2 reduced to CH_4 in the RPG effect was directly proportional to the amount of CH_3-S-CoM added, each micromole added resulting in the reduction of several micromoles of CO_2 to CH_4. We have studied the effect of CH_3-S-CoM concentration on both the rate of CH_4 formation and on CH_4 yield (moles of CH_4 formed per mole of CH_3-S-CoM added) in the RPG effect (Fig. 2). When the rate of methanogenesis from CH_3-S-CoM (H_2 atmosphere) was compared with the rate of methanogenesis in the RPG effect (H_2-CO_2 atmosphere), it was found that CO_2 increased the overall rate of methane formation. Previously, Romesser and Wolfe (10) used partially purified components of the methanogenic system, which catalyzed the reduction of CH_3-S-CoM but not of CO_2, to show that addition of CO_2 to the gas atmosphere increased the rate of

TABLE 1. Survey of methanogens for the presence of the RPG effect

Organism	Protein content (mg)	Rate of CH_4 production (nmol h^{-1} mg of protein^{-1}) with:[a]			Additional nmol of CO_2 reduced in RPG effect[b]
		CH_3-S-CoM + H_2	H_2 + CO_2	CH_3-S-CoM + H_2 + CO_2	
Methanobacterium bryantii	5.0	115	235	541	2,001
Methanospirillum hungatei	4.2	409	0	655	708
Methanobacterium formicicum	4.0	358	0	386	92
Methanobrevibacter ruminantium	5.0	288	0	228	24
Methanosarcina barkeri	4.8	105	77	72	0

[a] Each reaction mixture contained 37.5 μmol of PIPES buffer (pH 6.9), 1.0 μmol of ATP, 1.0 μmol of $MgCl_2$, 0.25 μmol of CH_3-S-CoM (where indicated), and 100 μl of cell extract in a volume of 165 μl.

[b] Each reaction was performed at 40°C and allowed to continue to completion. The data are corrected for background CH_4 formation in the absence of added substrate. This value varied among extract preparations, between 30 and 60 nmol of CH_4 being formed per 100 μl of extract during the reaction period.

CH_4 formation from CH_3-S-CoM. These results suggest that CO_2 is an allosteric effector of the methyl reductase.

At high concentrations of CH_3-S-CoM, at which the rate of methanogenesis was nearly maximal, the yield of CH_4 was directly proportional to the amount of CH_3-S-CoM added (Fig. 2), as previously reported (3). However, at low concentrations of CH_3-S-CoM, the CH_4 yield was inversely related to the initial concentration of CH_3-S-CoM added or to the observed rate of methanogenesis. This inverse relation of rate of methane formation and CH_4 yield also was found when substrates other than CH_3-S-CoM were used to induce the RPG effect (Table 2).

TABLE 2. Inducers of the RPG effect

Substrate[a]	CH_4 formation (nmol h^{-1} mg of protein^{-1}) in atmosphere of:[b]		Total CH_4 formed (nmol)[c]	
	H_2	H_2 + CO_2	H_2	H_2 + CO_2
CH_3-S-CoM	723	761	506	1,373
Formaldehyde	668	878	490	1,624
L-Serine	265	688	520	3,112
Sodium pyruvate	67	387	205	2,309
CH_3CH_2-S-CoM	0	356	0	1,212[d]

[a] Each reaction mixture contained 500 nmol of substrate, and each reaction was performed as described in the text.

[b] Data are corrected for background CH_4 measured in a control reaction lacking added substrate or CO_2 as noted in Table 1, footnote *b*.

[c] Measured after completion of the reaction.

[d] CH_3CH_2-S-CoM was reduced to C_2H_6.

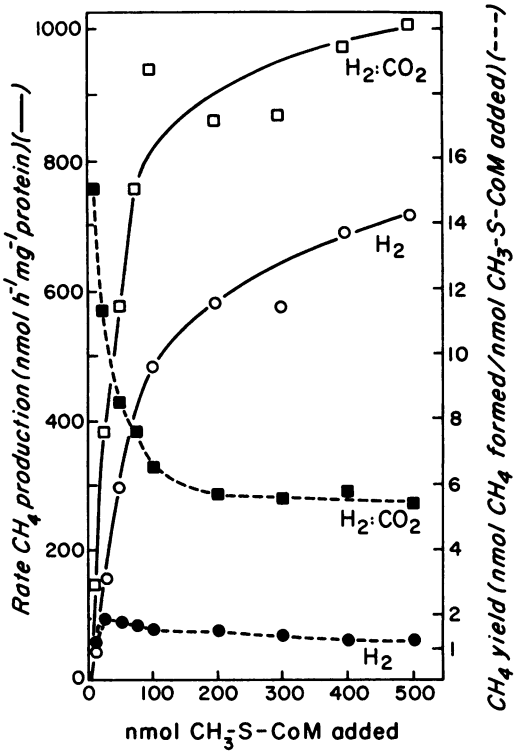

FIG. 2. Effect of CH_3-S-CoM concentration on methane formation in the RPG effect and in the methyl CoM reductase. Each reaction was performed as described in the text. The reaction volume was 400 μl. Activity of the methyl CoM reductase was measured in an H_2 atmosphere (●, ○). Activity of the RPG effect was measured in an 80% H_2:20% CO_2 atmosphere (■, □).

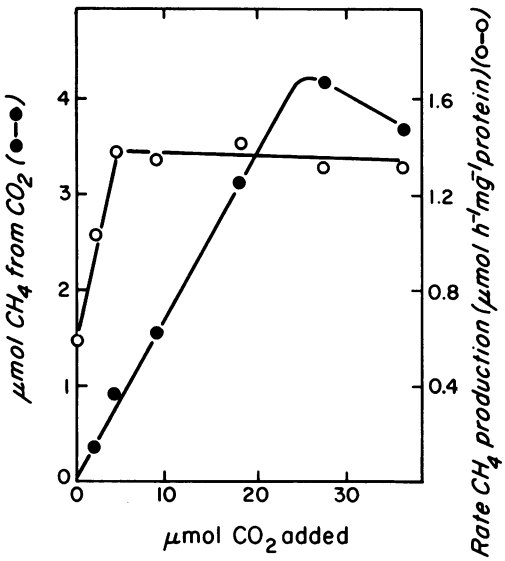

FIG. 3. Effect of CO_2 concentration on methane formation in the RPG effect. Each reaction was performed as described in the text. The reaction volume was 400 μl. The gas atmosphere was H_2; CO_2 was added in the amounts indicated.

CH_3-S-CoM and HCHO, which were rapidly reduced to CH_4, themselves gave CH_4 yields of 2.7 and 3.3, respectively, in the RPG effect. However, when compounds which themselves were reduced slowly were used to induce the RPG effect (e.g., serine or pyruvate), a high CH_4 yield was obtained (6 and 11 mol of CO_2 reduced per mol of substrate, respectively.)

The rate of reduction of the substrate inducing the RPG effect is not the sole factor to be considered, however. By measuring the appearance of C_2H_6 in the gas atmosphere, it was determined that cell extracts reduced 500 nmol of CH_3CH_2-S-CoM to C_2H_6 at the relatively slow rate of 145 nmol h^{-1} mg of protein^{-1}. Accordingly, a high CH_4 yield in the RPG effect was expected. The CH_4 yield obtained, however, was only 2.4. These results suggest that the CH_4 yield in the RPG effect is affected not only by the rate of reduction of the inducer but also by the concentration of CH_3-S-CoM (or CH_3CH_2-S-CoM) in the reaction mixture.

Effect of CO_2 concentration. Previously it was shown that addition of 500 nmol of CH_3-S-CoM to the reaction mixture resulted in maximal activity of the methyl reductase. When increasing amounts of CO_2 were added to the gas atmosphere (Fig. 3), it was found that the rate of methanogenesis increased, again supporting the idea that CO_2 acts as an allosteric effector for the methyl reductase. Maximal rates of methanogenesis were achieved in an atmosphere of approximately 2% CO_2. Maximal yield of methane from CO_2 was not obtained until the atmosphere was 10% CO_2, however. This suggests that CO_2 not only is an allosteric effector for the methyl reductase but also stabilizes the RPG effect.

Inhibition of the RPG effect. CoM derivatives inhibited both the methyl reductase and the RPG effect (Table 3), although inhibition of the RPG effect was greater. None of the CoM derivatives inhibited recovery of CH_4 from CH_3-S-CoM. However, those CoM derivatives that could be easily reduced to HS-CoM [$(S\text{-CoM})_2$ or CH_3CO-S-CoM] or which had sulfhydryl groups themselves significantly inhibited the efficiency of CO_2 reduction in the RPG effect.

Gunsalus et al. (2) demonstrated that HS-CoM (>10 mM) inhibited the rate of CH_3-S-CoM reduction by cell extracts. Similar inhibition of the methyl reductase was observed here (Fig. 4); however, the RPG effect was more strongly inhibited by HS-CoM. HS-CoM markedly decreased both the rate of methanogenesis and the amount of CO_2 reduced to CH_4. In a similar fashion, cysteamine (25 mM) had no effect on the methyl reductase, but decreased the rate of the RPG effect by 40% and the amount of CO_2 reduced to CH_4 by 60%. With 57 mM glutathione, the methyl reductase was not inhibited, but the RPG effect showed a 13% inhibition in rate and a 29% decrease in the amount of CH_4 produced.

DISCUSSION

McBride presented the first evidence of an RPG-like effect in cell extracts of a methanogen

TABLE 3. Inhibition of methanogenesis by CoM derivatives

Inhibitor	% Inhibition[a] with:			
	CH_3-S-CoM + H_2[b]		CH_3-S-CoM + H_2 + CO_2	
	Rate	Total CH_4	Rate	Total CH_4
$(S\text{-CoM})_2$	64.1	0	71.3	38.5
CH_3CO-S-CoM	39.4	0	62.6	23.9
$S{=}(CoM)_2$	5.5	0	47.6	2.6
$COOHCH_2$-S-CoM	33.3	0	33.3	0
3-Mercaptopropane-sulfonate	32.4	0	52.6	14.6
4-Mercaptobutane-sulfonate	20.1	0	54.5	26.3

[a] Each reaction mixture contained 48 μmol of PIPES buffer (pH 6.9), 2.5 μmol of ATP, 2.5 μmol of $MgCl_2$, 2.5 μmol of CH_3-S-CoM, 20 μmol of inhibitor, and 200 μl (9.2 mg of protein) of cell extract in 370 μl total volume. The data for total CH_4 were corrected for background CH_4 measured in a control reaction lacking the substrates CH_3-S-CoM or CO_2 as noted in Table 1, footnote *b*.

[b] Each reaction mixture was preincubated for 10 min at 60°C before CH_3-S-CoM was added.

(B. C. McBride, Ph.D. thesis, University of Illinois, Urbana, 1970). He found that in extracts of *M. bryantii* only 50% of the ^{14}C added as $^{14}CO_2$ was reduced to $^{14}CH_4$. However, when serine was added, 100% of the $^{14}CO_2$ was reduced to $^{14}CH_4$. This stimulation occurred whether serine was added initially or after the original CH_4 formation ceased. Acetate, pyruvate, glycine, glyoxalate, or glycolate could not replace serine. The results presented here with *M. thermoautotrophicum* clearly demonstrate that the presumed end products of CH_3-S-CoM reduction, CH_4 and HS-CoM or $(S\text{-}CoM)_2$, are not directly involved in the RPG effect. Instead, the finding that CH_3-S-CoM or CH_3CH_2-S-CoM, but not other CoM derivatives, stimulate CO_2 reduction suggest that the RPG effect is caused either by an allosteric effect of CH_3-S-CoM (or CH_3CH_2-S-CoM) or by generation of some previously undescribed chemical intermediate during the metabolism of CH_3-S-CoM. It appears that any compound which can itself be reduced to CH_4 also is able to induce an RPG effect in cell extracts of *M. thermoautotrophicum*. McBride and Wolfe (7) have shown that C-3 of serine and C-1 of pyruvate are reduced to CH_4 in cell extracts of *M. bryantii*. Metabolism of these methanogenic precursors is expected in *M. thermoautotrophicum*; Zeikus et al. (17) have demonstrated coenzyme A-dependent pyruvate dehydrogenase activity in extracts of *M. thermoautotrophicum*.

Any proposed model for the RPG effect must explain several features: (i) What is the nature of the stimulation event itself? (ii) By what mechanism is CO_2 reduction eventually stopped so that restimulation of CO_2 reduction is required? (iii) Why are sulfhydryl compounds effective inhibitors of the RPG effect? Finally, the model must be consistent with the observation that the yield of CH_4 from CO_2 is higher not only at high concentration of CO_2 but also when the rate of reduction of the inducer is slow or when low concentrations of CH_3-S-CoM are used.

At least two models appear to satisfy these requirements. In the first model, CH_3-S-CoM is required by cell extracts as an allosteric effector for CO_2 activation. In the second model, CH_3-S-CoM is metabolized by cell extracts to form a compound required for activation of CO_2. The allosteric model for the RPG effect requires not only that CH_3-S-CoM be a substrate for the methyl reductase but also be an effector for the binding or activation of CO_2 for its subsequent reduction to CH_4. In this system CO_2 would continue to be reduced as long as the rate of CH_3-S-CoM reduction was less than the rate of CO_2 activation, or as long as other methanogenic precursors were added to the extract to regenerate CH_3-S-CoM. If the K_m for CO_2 binding or activation were relatively high, then high CO_2 concentrations would be expected to improve CH_4 yield. Low concentrations of CH_3-S-CoM and the subsequent lower rate of CH_3-S-CoM reduction would also favor larger CH_4 yields. In the allosteric model CH_3CH_2-S-CoM might be expected to have sufficient chemical similarity to CH_3-S-CoM to provide allosteric activity. HS-CoM, on the other hand, might be a competitive inhibitor of CH_3-S-CoM. The sulfhydryls cysteamine and glutathione would be inhibiting in a different fashion, however, since they do not block the methyl reductase.

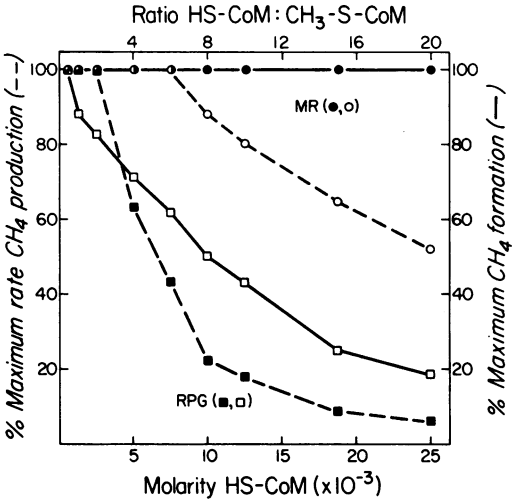

FIG. 4. Inhibition of the RPG effect and the methyl CoM reductase with HS-CoM. Each reaction was performed as described in the text. The reaction volume was 370 μl. Activity of the methyl CoM reductase (MR) was measured in an H_2 atmosphere (●, ○). Activity of the RPG effect was measured in an 80% H_2:20% CO_2 atmosphere (■, □).

The results presented here are also consistent with a model which proposes that the RPG effect is due to an unstable intermediate generated during the reduction of CH_3-S-CoM. The observed high CH_4 yield at high concentrations of CO_2 suggests that the RPG intermediate is stabilized by CO_2, perhaps by reaction with it. The intermediate is strongly inhibited by sulfhydryl compounds and may be self-quenching, since at high concentrations of CH_3-S-CoM, at which high concentrations of the intermediate would be expected, the observed CH_4 yield was low.

One type of compound which would satisfy these observations is a free radical. For example, the thiyl free radical of CoM, · S-CoM, could be generated in the reduction of CH_3-S-CoM

$$CH_3\text{-}S\text{-}CoM + H_2 \rightarrow \cdot\ S\text{-}CoM + H\cdot + CH_4.$$

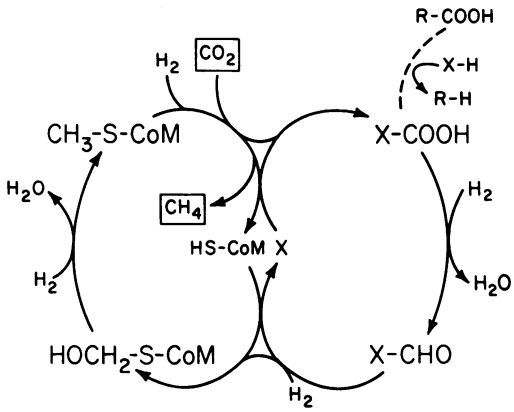

FIG. 5. Proposed model for the RPG effect and for the reduction of CO_2 to CH_4 in extracts of *M. thermoautotrophicum*.

The CoM thiyl free radical formed could then react with CO_2 directly or, alternatively, · S-CoM could abstract a proton from another molecule to form a new free radical which could react with CO_2. Formation of a free radical would allow part of the energy derived from the reduction of CH_3-S-CoM with H_2 to be conserved in the activation of CO_2. A candidate for an alternative carrier may be methanopterin, a pterin molecule recently identified by Keltjens and Vogels (4), which appears to carry a C_1 unit at the carboxyl level of oxidation. John Leigh (personal communication) found that methanopterin was required for methanogenesis from CO_2 by resolved cell extracts of *M. thermoautotrophicum*, but he did not determine that it actually carried a C_1 moiety.

If a free radical is involved in the RPG effect, competing reactions such as disulfide formation or condensation of the thiyl radical and the hydrogen atom to form HS-CoM could account for the observed instability of the RPG intermediate. Similarly, it would be predicted that the addition of free radical scavengers to the system would reduce the rate of methanogenesis from CO_2 and decrease the amount of CO_2 reduced to CH_4. These predictions are consistent with the observations made when sulfhydryls, such as HS-CoM, cysteamine, glutathione, or 3-mercaptopropanesulfonate, were added to cell extracts. Sulfhydryls are known to be good free radical scavengers (5), presumably acting by a process of hydrogen donation.

The Barker scheme (1) for the reduction of CO_2 to CH_4 has been reexamined in view of the observations made on the RPG effect in cell extracts of *M. thermoautotrophicum*. A pathway for CO_2 reduction in this methanogen is proposed (Fig. 5). As in the Barker model, the C_1 moieties are bound throughout, CoM being a carrier at the methanol and formaldehyde levels of oxidation; methanopterin may be a carrier at the formate and carboxyl levels (4). Reduction of CH_3-S-CoM and activation of CO_2 are coupled reactions. In this pathway, the RPG effect would be elicited by adding any methanogenic precursor to cell extracts, including those which enter the pathway via decarboxylation of RCOOH (e.g., pyruvate). This pathway is proposed only as a working hypothesis for future experimentation. Blocking the RPG effect with spin-trapping reagents, generating free radicals by photolysis or chemical means, or demonstrating free radical formation by electron spin resonance may be useful techniques for evaluating intermediates in the RPG effect.

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