Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the rate-limiting and final step in methane biosynthesis. Using coenzyme B as the two-electron donor, MCR reduces methyl-coenzyme M \((\text{CH}_3\text{SCoM})\) to methane and the mixed disulfide, CoBS-SCoM. MCR contains an essential redox-active nickel tetrahydrocorphinoid cofactor, Coenzyme F_{ads}, at its active site. The active form of the enzyme (MCR_{red1}) contains Ni(II)-F_{430}. Rapid and efficient conversion of MCR to MCR_{red1} is important for elucidating the enzymatic mechanism, yet this reduction is difficult because the Ni(II) state is subject to oxidative inactivation. Furthermore, no in vitro methods have yet been described to convert Ni(II) forms into MCR_{red1}. Since 1991, it has been known that MCR_{red1} from Methanobacterium marburgensis can be generated in vivo when cells are purged with 100% H_{2}. Here we show that purging cells or cell extracts with CO can also activate MCR. The rate of in vivo activation by CO is about 15 times faster than by H_{2} (130 and 8 min^{-1}, respectively) and CO leads to twofold higher MCR_{red1} than H_{2}. Unlike H_{2}-dependent activation, which exhibits a 10-h lag time, there is no lag for CO-dependent activation. Based on cyanide inhibition experiments, carbon monoxide dehydrogenase is required for the CO-dependent activation. Formate, which also is a strong reductant, cannot activate MCR in M. marburgensis in vivo.

Mathanogenes are responsible for all biological methane production on earth, generating 10^{7} tons of methane annually. Being strict anaerobes, they are widely distributed in anoxic environments where electron acceptors such as NO_{3}^{-}, Fe^{3+}, and SO_{4}^{2-} are limiting, such as aquatic sediments, the digestive tract of animals, rice fields, sewage digesters, landfills, heart wood of living trees, and decomposing algal mats (Garicu et al., 2000). Methanogens play critical roles in the carbon cycle by converting products from anaerobic fermentation (such as hydrogen, carbon dioxide, methanol, formate, and acetate) into methane (Thauer, 1998; Hanson, 1996). Only by such means can methanogens obtain energy to grow (Thauer, 1998; Ferry, 2010). Methane, the principal component of natural gas, is an important source of clean renewable energy, with the highest heat production per mass unit (55.7 kJ g^{-1}) among all hydrocarbons (Richard and Ball, 2008). However, methane also is a potent greenhouse gas (Hanson and Hanson, 1996) and, predominantly due to changing agricultural practices (e.g., increased development of rice production and livestock cultivation) over the past two centuries, the atmospheric concentration of methane has more than doubled, reaching a level (1779 ppb in 2005) that far exceeds the natural range (320–790 ppb) of the last 650,000 years (Wuebbles and Hayhoe, 2000). Therefore, because of the effect of greenhouse gases on climate change, understanding the basis of methane production is an important research goal, while controlling methane emissions is an important aim for governmental policy makers.
Wolfe, 1983). However, only 1–5% of the MCR activity can be isolated in a much more active form (up to 50% activity) when MCR could be isolated in a much more active form (up to 50% activity) when

The MCR catalyzes the oxidation of HSCoM to H2 and CO2. The catalytic mechanisms for MCR-catalyzed reaction have been under debate for a long time. These two mechanisms in principle differ in how they describe the initial cleavage of the sulfur–carbon bond of the substrate CH3-SCoM (Ermler, 2005). Since none of the proposed intermediates have been trapped and spectroscopically or structurally characterized when using natural substrates, both mechanisms remain controversial. Based on spectroscopic and rapid kinetic studies, a hybrid mechanism involving both methyl-Ni and methyl radical has been proposed (Dey et al., 2010).

The concentrations of Ti(III) citrate were determined routinely by spectrophotometry or structurally characterized when using natural substrates. Both mechanisms remain controversial. Based on spectroscopic and rapid kinetic studies, a hybrid mechanism involving both methyl-Ni and methyl radical has been proposed (Dey et al., 2010).

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It is rather straightforward to purify native MCR from methanogenic cells; however, it has been extremely difficult to isolate and maintain MCR in its active state, due to the low midpoint redox potential of the Ni(II)/Ni(I) couple of bound F430 (Kunz et al., 2006) with a slight modification of the sulfur and acetate concentrations. Enzyme extracts of M. thermoautotrophicum were used to activate MCR from Methanothermobacter thermautotrophicus in vitro (Gunsalus and Wolfe, 1980; Nagle and Wolfe, 1983). However, only 1–5% of the MCR activity can be recovered by this activation.

In 1991, Thauer and colleagues found that the M. marburgensis MCR could be isolated in a much more active form (up to 50% of the in vivo activity) when cells are pre-incubated with 100% H2 and CH3-SCoM (or H2SCoM) before lysis and purified under strict anaerobic conditions in the presence of Ti(III) citrate and HScCoM. CH3CoM and HScCoM were used to stabilize both the activity and the EPR signal of MCRred1 (Rospert et al., 1991a). Since then, H2-dependent activation has been used to purify MCRred1 and 50–90% MCRred1 is routinely obtained (Li et al., 2010; Duin et al., 2011). Thus, although the steps involved in MCR activation are not known, it is clear that MCR activation involves a redox activation to the Ni(II) state (Rospert et al., 1991b; Dey et al., 2006).

Here we report that carbon monoxide, which is a stronger reductant than H2, can activate MCR in vivo and in cell extracts of M. marburgensis. The rate of CO-dependent activation (130 min−1) is about 15-fold faster than H2-dependent activation (8 min−1). Furthermore, CO can activate MCR to a higher percentage. We observe no activation of MCR when cells are treated with formate, another strong reductant, using similar conditions as with CO or H2.

MATERIALS AND METHODS

BIODECHEMICALS, GAS MIXES, AND CELL GROWTH

All buffers, media ingredients, and other reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA) and, unless otherwise stated, were of the highest purity available. Solutions were prepared using Nanopure deionized water. N2 (99.99%), CO (99.99%), argon (99.6%), H2/CO2 (80%/20%), and ultrahigh purity H2 (99.999%) were obtained from cryogenic gases (Grand Rapids, MI, USA). Ti(III) citrate solutions were prepared from a stock solution of 200 mM Ti(III) citrate, which was synthesized by adding sodium citrate to Ti(III) trichloride (30 wt% solution in 2 N hydrochloric acid; Acros Organics, Morris Plains, NJ, USA) under anaerobic conditions and adjusting the pH to 7.0 with sodium bicarbonate (Zehnder and Wohlgemuth, 1976). The concentration of Ti(III) citrate was determined routinely by titrating a methyl viologen solution. M. marburgensis (formally M. thermoautotrophicum strain Marburg) was obtained from the Oregon Collection of Methanogenics catalog as OCM82. M. marburgensis was cultured on H2/CO2 (80%/20%) at 65°C in a 14-L fermentor (New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA). Culture media were prepared as previously described (Kunz et al., 2006) with a slight modification of the sulfur and reducing source, by adding 50 mM sodium sulfide (instead of H2S gas) at a flow rate of 1 ml min−1 during the entire growth period.

ACTIVATION OF MCR IN WHOLE CELLS BY H2, CO, OR FORMATE

Solutions were prepared and all manipulations were performed under strictly anaerobic conditions in a Vacuum Atmospheres (Haworth, CA, USA) anaerobic chamber maintained under nitrogen gas at <1 ppm of oxygen (monitored continuously by a Teledyne detector). Cells were grown under H2/CO2 as described (Dey et al., 2010a) and, before harvest, the cells were treated with H2 for 30 min in the fermenter (different gases were used in the experiments shown in Figure 1D). Then, the cells were harvested, transferred to the anaerobic chamber, and resuspended in 50 mM Tris, pH 7.6, 10 mM HSCoM, 0.1 mM Ti(III) citrate, and aliquoted into 150 ml serum-stoppered anaerobic bottles, as described (Dey et al., 2010a; Li et al., 2010). The headspaces of the vials containing the resuspended cells were then purged with CO or H2 for 10 min, or at timed intervals right before samples were taken for analysis. This second (and all subsequent) purge was also omitted for the formate-activation experiment as described in the next paragraph.
Zhou et al. Activation of MCR by CO

FIGURE 1 | Activation of MCR by H2 and CO in vivo. (A) EPR spectra of the H2-activated cell samples at different time points; (B) EPR spectra of the CO-activated cell samples at different time points; (C) plot of intensity of the MCRred1 EPR signal (the "S"-shaped derivative signal at ≈3250 G, double-sided arrow) over time with duplicate H2 (circles) and CO (triangles) activated cell samples; (D) plot of MCRred1 EPR intensity over time with CO activation with no initial purge (circled) in the fermentor; or initially purged with H2 (triangle), CO2 (square), or CO (diamond) in the fermentor; (E) plot of MCRred1 EPR intensity over time of treatment (as in C) with H2 (circles) and CO (triangles), arrow indicates the switch of gases. The features of the MCRox1 and MCRred1 signals are depicted with arrows to demarcate their g values.

to ensure that the gas treatment did not interfere with the formate incubation. To facilitate equilibration of the gas with the solution, the vials were shaken every 2 min. In most experiments, the cell suspension was transferred into smaller vials, where the headspace was purged again for 10 min with the test gas. Purges with H2 were done inside of the chamber, while, for safety reasons, purges with CO were done in the fume hood outside of the chamber. To remove trace amounts of oxygen in the H2 or CO line for aerating the serum vials, the line was fitted with a high pressure Oxy-Trap oxygen scrub (Alltech, Deerfield, IL, USA) followed by an indicating Oxy-Trap (Alltech) to monitor the efficiency/remaining capacity of the oxygen scrub. Between the purges, the serum vials were inverted in a water bath in the anaerobic chamber to minimize any gas escape. For monitoring the conversion of MCR to the MCRred state, 200 μl samples were removed at different time points to be analyzed by EPR spectroscopy.

To determine whether formate activates MCR, the same protocol outlined above was followed (including the first 30 min H2 purge in the fermentor), except that sodium formate was added directly to the cell suspension to a final concentration of 100 or 500 mM (pH did not change in the Tris buffer that is described above for resuspending cells). Thus, there was only one gas purge in this experiment. To inhibit carbon monoxide dehydrogenase (CODH) activity, NaN3 was added to the cell suspension to a final concentration of 0.2 or 0.4 mM before aerating with CO. Then, the cell suspension was incubated as described above.

SPECTROSCOPY OF MCR
UV-visible spectra of MCR were recorded in the anaerobic chamber using a diode array spectrophotometer (model DT 1000A, Analytical Instrument Systems, Inc., Flemington, NJ, USA). All samples for EPR spectroscopy were prepared in 50 mM Tris–HCl buffer, pH 7.6, in a Vacuum Atmospheres anaerobic chamber maintained under nitrogen gas at <1 ppm of oxygen. EPR spectra were recorded on a Bruker EMX spectrometer (Bruker Biospin).
Corp., Billerica, MA, USA), equipped with an Oxford ITC4 temperature controller, a Hewlett-Packard model 3540 automatic frequency counter, and Bruker gaussmeter. Unless otherwise noted, the EPR spectroscopic parameters were: temperature, 70 K; microwave power, 10 mW; microwave frequency, 9.43 GHz; receiver gain, 2 × 10^4; modulation amplitude, 10 G; and modulation frequency, 100 kHz. Deoxygenated cell extracts of the EPR spectra were performed and referenced to a 1-mM copper perchlorate standard.

**PROTEIN PURIFICATION**

The purification of MCRred1 from *M. marburgensis* was performed under strictly anaerobic conditions, as described previously (Kunz et al., 2006). The amount of MCRred1 was calculated according to the UV-visible and EPR spectra (Kunz et al., 2006; Li et al., 2010). Protein concentration was determined by Rose Bengal assay (Elliott and Brewer, 1978) with lysozyme as the standard.

**ENZYME ACTIVITY ASSAYS**

Methyl-coenzyme M reductase assays for methane formation from methyl-CoM and CoBSH were performed at 60°C, basically as described by Kunz et al. (2006). The standard assay mixture contained 10 mM [14C]CH3-SCoM, 0.1 mM CoBSH, 1.8 mM aquacobalamin, 20 mM Td(TIII) citrate, and 0.5 mM Tris (pH 7.2) in a final volume of 0.2 ml. The reaction was started by adding MCR. MCR activity was measured by following the time-dependent loss of radioactivity from the methyl group of [14C]CH3-SCoM, which is converted to highly volatile and insoluble [14C] methane (Kunz et al., 2006). Rates of methane formation were calculated from the linear portion of the time course. One unit of MCR activity is equal to 1 μmol of methane produced per minute.

To determine formate dehydrogenase (FDH), hydrogenase, and CODH activities, *M. marburgensis* cell extracts were prepared under anaerobic conditions as described (Kunz et al., 2008). All assays were performed in a 1-ml sealed solution at 60°C. The anaerobic assay mixture contained 20 mM methyl viologen, 50 mM Tris, pH 7.5, and cell extract. For the hydrogenase and CODH assays, oxygen-free hydrogen gas or carbon monoxide (99.9%), respectively, was bubbled into the assay mix in a serum-vial. Activity was measured by following the reduction of methyl viologen at 578 nm with an extinction coefficient of 9.78 mM⁻¹ cm⁻¹ or the reduction of NADPH⁺ at 340 nm with an extinction coefficient of 6.27 mM⁻¹ cm⁻¹.

**RESULTS**

**GENERATION OF MCRred1 IN VIVO BY CO**

To date, the most effective way to activate MCR has been to incubate the cell suspension under a H2 atmosphere (Rospert et al., 1994a, Kunz et al., 2006). While this is effective for generating MCRred1 from organisms like *M. marburgensis*, for some methanogens, like *Methanoarcus aerivorans*, H2 activation is inefficient because the hydroxylases were weakly expressed (Guss et al., 2009; Wang et al., 2011). Therefore, there must be other pathways for in vivo activation of MCR. We hypothesized that the low-potential reductant, CO, with a standard reduction potential (vs. NHE for the CO/CO couple) of −520 mV (in comparison to −420 mV for that of 2 H²/H2; Lehn and Ziesied, 1982) should be able to activate MCR.

To measure the effectiveness of CO in activating MCR, we purged the *M. marburgensis* culture for 30 min with H2 in the fermentor, harvested the cells, resuspended the cells in buffer and incubated the cell suspensions at 32°C in a water bath under a CO or H2 atmosphere. The whole-cell EPR spectra of the H2- and CO-treated cells at different time points after initiating H2 or CO gas purge are compared in Figures 1A,B, respectively. The activity of MCR is linearly related to the MCRred1 EPR signal, with g values at 2.24 and 2.065; thus, EPR spectroscopy is a convenient method to assess the level of MCR activation achieved. It also reveals various other EPR-active forms of MCR that are present during the treatment – for example, MCRred1, which is an oxidized form of MCR that has a distinct EPR spectrum (g values at 2.23 and 2.16), can be converted into the MCRred2 state by the addition of low-potential reductants like Td(TIII) citrate, and MCRred2, a Ni(I) state that is formed when the enzyme in the MCRred1 form is treated with HSCoM and CoBSH (Mahlert et al., 2002). Therefore, we feel that assessing the level of MCRred1 is the most accurate indicator of the level of activation achieved. The spectra of the cell suspension before the CO/H2 purge reveal about two to three times more MCRred1 than MCRred2, very little MCRred2, and the remainder in the EPR-silent Ni(II) form. For the H2-purged cells, after ~1 h of incubation, MCRred1 disappeared as MCRred2 increased; then, over the next ~20 h, MCRred2 appeared. Following a ~10-h lag time, the rate constant for H2-dependent activation was 8 min⁻¹. The amount of MCRred1 formed at the end of activation, when compared with the amount of MCRred1 in the beginning, varied slightly among enzyme preparations.

In contrast, when cells were incubated with CO during the time purge, within 1 h, MCR underwent rapid activation to the MCRred1 state without a noticeable lag time or intermediacy of MCRred2. The enhanced activation by CO was noticeable by eye – the cell suspension began turning green immediately after aerating with CO. However, for H2-dependent activation, this color development required overnight incubation. At the end of activation (when the whole-cell EPR signal reached maximum intensity), the CO-activated cell suspension was also greener than that of the H2-activated cells (data not shown). Quantitative analysis of the activation confirmed the visual observations. The rate constant for CO-dependent activation was 130 min⁻¹, which is over 15-fold faster than that observed for activation by H2; furthermore, the amount of accumulated MCRred1 was ~twofold higher than with H2 (Figure 1C). Furthermore, with CO activation, the amount of MCRred1 formed at the end of activation was approximately two times more than the initial amount of MCRred1, indicating that CO activation converts the MCRred1 form to MCRred1.

As described above, the CO-dependent activation experiments shown in Figures 1B,C involved an initial 30 min H2 purge in the fermentor. To test whether the initial H2 treatment is required, we purged the cell suspension with pure H2, CO, or CO2 in the fermentor, or omitted the initial 30-min purge before harvesting.
and then bubbled the cell suspension with CO after resuspending the cells in Tris–HCl buffer as above. The initial EPR spectra significantly differed with different purge conditions. MCRox1 was only observed when the cells were initially purged with H2 in the fermentor, while MCRred1 was only noticeable with the CO treatment or when the initial H2 purge was omitted. On the other hand, MCR appears to be completely in a Ni(II)-silent form when cells were initially purged with CO2, because no detectable MCR signal was present. However, MCR underwent activation in all these conditions (i.e. H2, CO, CO2, no initial purge) after treating the cell suspensions with CO, and the activation rates and the final levels of activation were similar (Figure 1D). Thus, an initial H2 purge is not required for CO-dependent activation of MCR. This experiment also clearly shows that the EPR-silent Ni(II)-MCR forms undergo conversion to MCRred1 by CO in vivo.

We also tested the effect of addition of CO to the H2-purged cells. Figure 1E shows that treatment with CO can further activate MCR in H2-purged cells to nearly reach the level of MCRred1 observed in CO-activated cells. On the other hand, treatment with H2 does not increase the MCRred1 level in CO-activated cells. These experiments indicate that H2- and CO-dependent activation pathways converge after the initial transfer of electrons to the acceptor of reducing equivalents from hydrogenase or CODH.

EFFECT OF TEMPERATURE ON CO-DEPENDENT MCR ACTIVATION
H2-dependent activation of MCR is very sensitive to the incubation temperature; it is most effective at 30–33°C (Kunz et al., 2006). To test whether CO-dependent MCR activation is also temperature-dependent, we incubated the cell suspension with CO at different temperatures and followed the whole-cell EPR spectrum over 12 h. As shown in Figure 2, CO-dependent activation, like the H2-dependent process, is most effective at ~30°C. The complicating factor in these experiments is the inactivation of MCR, which occurs markedly faster as the temperature increases (Horng et al., 2001). At high temperatures (40–60°C), MCRred1 decays after 2 h of CO-dependent activation, while, at lower temperatures (10–20°C), activation by CO occurs more slowly, but, because MCRred1 decays more slowly, this form of the enzyme is more stable and accumulates to higher levels.

CODH ACTIVITY REQUIREMENT FOR CO-DEPENDENT ACTIVATION OF MCR
To test whether CODH activity is involved in CO-dependent activation of MCR, CN−, which is a competitive slow-binding inhibitor of CODH with a Ki for CODH inhibition in the low micromolar range (Ragsdale et al., 1983; Ha et al., 2007), was added to the cell suspension. As shown in Figure 3, 0.2 mM CN− blocked the CO-dependent activation of MCR, indicating that CODH activity is required for CO-dependent activation of MCR. In contrast, 0.2–0.4 mM CN− does not block H2-dependent activation of MCR (data not shown). We measured a specific activity of 3–5 U mg−1 of CODH in cell extracts, which is significantly lower than that of hydrogenase (~80 U mg−1), but is sufficient to drive the activation of MCR.

FORMATE CANNOT ACTIVATE MCR
The standard redox potential of the CO2/HCOOH half-cell (~430 mV, Eq. 2) is equivalent to that of the 2H+/H2 half-cell (Eq. 3; Scheer and Thauer, 1978). Furthermore, the FDH gene (fdh) is present in the M. marburgensis genome and, based on
sequence identity, is thought to use F420 as an electron donor (Liesegang et al., 2010; Kaster et al., 2011a). Thus, we expected that formate could also activate the *M. marburgensis* MCR. However, as shown in Figure 5, we did not observe any increase in the level of MCRred1 upon incubation of the cell suspension for over 20 h with formate. Correspondingly, we measured only a very low specific activity of FDH in cell extracts of *M. marburgensis* (3–4 mU mg\(^{-1}\)), using NADP\(^+\) as electron acceptor and 2–3 mU mg\(^{-1}\), using MV), which is consistent with earlier reported values of 3–8 mU mg\(^{-1}\), depending on the electron acceptor and the pH of the assays (Tanner et al., 1989). We assayed using both NADP\(^+\) and MV, which is an electron acceptor for the F420-dependent enzyme, which the *M. marburgensis* enzyme is predicted to be (Kaster et al., 2011a). There are two forms of FDH: a tungsten- and a molybdenum-containing enzyme. The growth medium that we used has 1 μM molybdenum, but no tungsten (Kunz et al., 2006); however, addition of 1 μM tungsten and 1.1 μM selenium to the growth medium for *M. marburgensis*, also did not lead to activation of MCR in the presence of formate (data not shown). Apparently, the low level of FDH activity present in *M. marburgensis* is not sufficient to drive activation of MCR.

\[
\begin{align*}
\text{CO}_2 + 2\text{H}^+ + 2e^- & \rightarrow \text{HCOOH} \quad E^0 = -0.43 \text{ V} \\
2\text{H}^+ + 2e^- & \rightarrow \text{H}_2 \quad E^0 = -0.42 \text{ V} \\
\text{CO}_2 + 2\text{H}^+ + 2e^- & \rightarrow \text{CO} + \text{H}_2\text{O} \quad E^0 = -0.52 \text{ V}
\end{align*}
\]

**DISCUSSION**

In order to study the mechanism of methane formation, it is crucial to develop an effective protocol for activation of MCR, the rate-limiting enzyme in this process. MCR has a number of different states, some of which have been detected in vivo (Albracht et al., 1988; Rospert et al., 1991a; Becker and Ragsdale, 1998) and others that have been observed in vitro (Mahlert et al., 2002; Finazzo et al., 2003; Kern et al., 2007; Harmer et al., 2008). Among these forms, only the NE(I)-MCRred (red1 and red2) forms are active and there is evidence supporting (Kunz et al., 2006; Dey et al., 2007, 2010a) and questioning (Ghosh et al., 2001; Pelmenschikov et al., 2002; Pelmenschikov and Siegbahn, 2003) the catalytic relevance of the methyl-Ni(III) state.

Without treating cells before they are harvested, MCR is isolated in the inactive Ni(II) state. This is the stable state of the enzyme and, except for one structure of the methyl-Ni(III) state (Cedervall et al., 2011), it is the only form whose crystal structure is known. There exist no published methods for conversion of any of the Ni(II)-MCRsilent forms into MCRred1 in vitro. Thauer and colleagues discovered that MCRox1, an inactive EPR-active state that has been termed the "ready" state, can be generated by purging the cells with 80% N\(_2\)/20% CO\(_2\) before harvesting; then MCRox1 can be isolated and reduced to active MCRred1 in vitro by addition of the reductant, Ti(III) citrate (Goubeaud et al., 1997). In lieu of the gas purging protocol, formation of MCRred1 can be optimized by adding sulfide to the culture prior to harvesting (Becker and Ragsdale, 1998). Then, as with the gas-switching method, the relatively stable MCRred1 state of the enzyme can be purified and quantitatively converted to MCRred1 by treating with Ti(III) citrate.
Here we show that CO, like H2, can activate the \textit{M. marburgensis} MCR in vivo. When cells are purged with CO (Figure 1C), the whole-cell EPR signal of MCRred1 increases much more rapidly than that of H2-purged cells. The more rapidly MCR can be activated, the better, because oxidants are present in the whole cells and in extracts that oxidize the Ni(I) state back to inactive Ni(II). However, once the enzyme is purified and maintained under anaerobic conditions, the MCRred1 state is rather stable. This CO-purging protocol yields purified MCR that is 80–90\% MCRred1, as measured by the UV-visible and EPR spectral intensities and the specific activity.

Because growth of \textit{M. marburgensis} on CO has not been reported, we were somewhat surprised that CO can activate MCR. However, there is a copy of the CODH gene (MTBMA e02870-02930) in the \textit{M. marburgensis} genome (Liesegang et al., 2010; Kaster et al., 2011a) and relatively high CODH activities are measured in the cell extracts. This enzyme would be required for the autotrophic fixation of CO2 by \textit{M. marburgensis} (Ferry, 2010). It is likely that it is this CODH activity that is required for CO-dependent activation of MCR, because we are unaware of other enzymes that can use CO as an electron donor and because CN–, a potent CODH inhibitor, blocks CO-dependent activation of MCR (Figure 3).

On the other hand, incubation of cells with formate under the same condition used for H2- and CO-dependent activation does not activate MCR. Although \textit{M. marburgensis} can assimilate formate and has FDH activity, it is unlike the related strains, \textit{Methanothermobacter thermautotrophicus} strain Z-245 and \textit{Methanothermobacter wolfeii} (Nolling et al., 1995; Wassfallen et al., 2000), in that it cannot grow on formate as an energy source (Kaster et al., 2011a). Mutants of \textit{M. marburgensis} lacking FDH require formate for growth on H2 and CO2 (Tanner et al., 1989). Our experiments confirm earlier reports (Tanner et al., 1989) that the specific activity of FDH is very low — approximately 1000-fold lower than that of CODH, probably insufficient to support activation of MCR.

Thus, there are now two known electron sources that can reduce MCR to active MCRred1: H2 and CO. One possible activation mechanism involves H2 as an intermediate because CO-dependent H2 production has been reported in methanogens (Daniels et al., 1977; Olliven et al., 1994), however, it is clear that this is not the pathway for CO-dependent activation of MCR because the rate of activation by CO is about 15 times faster than that with H2. Furthermore, the lag phase observed in H2-dependent activation is not present in the CO-dependent process. One might also argue that CO is an intermediate in the H2-dependent activation process and that the lag phase observed in H2-dependent activation is due to the time needed for formation of CO from H2 and CO2 in the suspensions. However, this possibility is ruled out by the fact that CN– blocks CO2, but not H2-dependent activation of MCR. Yet, CO further activates the H2-activated enzyme to reach the level of MCRred1 observed in CO-activated cells (but not vice versa — H2 hardly activates MCR in CO-activated cells) indicates that H2- and CO-dependent activations pathways converge after the initial electron transfer from hydrogenase or CODH. The reason for the faster and higher activation level of MCR by CO may be related to the standard redox potential of the CO2/CO half-cell (−520 mV, Eq. 4), which is approximately 100 mV more negative than that of the 2H+/H2 half-cell (Lehn and Zassel, 1982; Drake et al., 2006; Thauer et al., 2008; Kaster et al., 2011b). According to Marcus theory, for a one-electron redox reaction, a 100-mV greater driving force would be expected to increase the rate of electron transfer by ~30-fold at 30°C. Accordingly, we observe that CO-dependent activation is about 15 times faster that the H2-dependent process. This driving force argument is also supported by our results that CO activation is faster than H2 even though hydrogenase activity in \textit{M. marburgensis} cells is 20-fold greater than that of CODH.

Activation of MCR involves reduction of the nickel center of FeoB to the Ni(II) state (Rospert et al., 1999b; Dey et al., 2006). The electron carrier(s) needed for activation must interface with CODH (or hydrogenase, when cells are activated with H2) and with MCR. Although the methanogenic CODH is recognized to interface with ferredoxin (Tersely and Ferry, 1988), our results indicate that there are additional redox steps involved in activation that couple CO-reduced ferredoxin to MCR. These components are probably also required in the H2-dependent activation. However, the activation by H2 is likely to be more complex than that for CO because the midpoint potential for the Ni(II)/Ni(IV) couple of bound FeoB (less than −600 mV) is significantly lower than that of ferredoxin (approximately −450 mV) and 2 H+/H2 (−414 mV), and only slightly lower than that of CO2/O2 (−520 mV). Thus, some type of bioenergetic coupling is required for activation of MCR (at least in the case of H2-dependent activation). We speculate that this mechanism of activation might involve electron bifurcation, which has been seen to drive various uphill enzymatic reactions (Liu et al., 2012; Thauer, 2012).

Cell extracts of \textit{M. thermoautotrophicus} have been shown to slowly catalyze the reduction of CO2 to methane only upon addition of CH4-SG-Mer CoBS-SGoM (Gumahara and Wolfe, 1977; Rouvière and Wolfe, 1988). This phenomenon is referred to as the RPF effect, which couples the first step in methanogenesis from CO2 and H2 to the reduction of CoBS-SGoM to the free thiolate cofactors. Kaster et al. (2011a) demonstrated that the MvhADG/HdAABC complex from hydrogenotrophic methanogens couples the endergonic reduction of ferredoxin (midpoint potential of −450 to −500 mV) with H2 to the exergonic reduction of CoBS-SGoM with H2. Furthermore, reduction of ferredoxin by H2 (−414 mV at 100% gas phase under 1 bar pressure) at pH7 occurs only if CoBS-SGoM is present — thus, this is a coupled and energy driven reaction. We speculate that electron bifurcation involving CoBS-SGoM may be involved in the uphill reductive activation of MCR. The need for coupling of reduction of ferredoxin to the reduction of CoBS-SGoM by H2 may explain why there is a lag phase in the H2-dependent activation of MCR.

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REFERENCES
Albracht, S. P. J., Ankel-Fuchs, D., Becher, B., Ehemann, J., Joll, M., Van Der Zwan, J. W., et al. (1988). Fine
new EPR signals assigned to nickel in methyl-coenzyme M reductase from Methanobacterium thermoauto-
trophicum, strain Marburg. Biochim. Bio-
phys. Acta 955, 86–102.
Becker, D. F. and Ragdale, S. W. (1998). Activation of NCd-MCoR reduct-
tase to high specific activity after treatment of whole cells with sodium sulfide. Biochemistry 37, 2679–
2687.
Brenner, M. C., Zhang, H., and Scott, R. A. (1995). Nature of the low activity of S-methyl-coenzyme M reductase as
determined by active site titrations. J. Biol. Chem. 268, 14041–14045.
Cederwall, P. E., Dej, M., Li, X., Sarangi, R., Hedman, B., Ragdale, S. W., et al. (2011). Structural analysis of a 5-S-methyl protein in methyl-
coenzyme M reductase from Methano-
thermotrophiaceae. J. Am. Chem. Soc. 133, 5626–5628.
Cederwall, P. E., Dej, M., Pearson, A. R., Ragdale, S. W., and Wilcox, C. M. (2010). Structural insight into methyl-coenzyme M reductase chemistry using coumarin B analogs. Biochemistry 49, 7683–
7693.
Dannals, L., Fuchs, G., Thauer, R. K., and Zilisch, J. G. (1977). Carbon monox-
ide inhibition by oxygen and diaz-
toia. J. Bacteriol. 132, 118–128.
Dej, M., Kunz, R. H., Hua, K. M., V., Craft, J. L., Horng, Y .-C., Tang, Q., et al. (2006). Spectroscopic and crystallographic study of red-
tation of the metal versus the tetrapyrrole
substrate analogue. Biochemistry 45, 7653–7676.
Dener, S., Jia, M., Guns Rich, M., Thauer, R., and Harmen, J. (2010). Binding of coenzyme B induces a major conformational change in the active site of methyl-coenzyme M reductase. J. Am. Chem. Soc. 132, 567–575.
Elliott, J. J., and Breslow, E. M. (1978). The induction of protoporphyrin by 2,3-butanedioic acid. Arch Biochem.
Biphys. 190, 351–357.
Enders, U. (2005). On the mechanism of methyl-coenzyme M reductase: Defl-
aton D. J., and Enders, U. (2005). How to make a lore by exhalation methane. Annu. Rev. Microbiol. 64, 453–473.
Dej, M., Kunz, R., Lynn, D. M., and Ragdale, S. W. (2007). Character-
ization of Ni-nickel sulfides generated by reaction of methyl-
coenzyme M reductase with bromi-
nated acids. Biochemistry 46, 11949–
11978.
Dej, M., Li, X., Kanz, R. C., and Ragdale, S. W. (2010a). Detection of organometallic and radical inter-
mediates in the catalytic mechanism of methyl-coenzyme M reductase using the natural substrate methyl-
coenzyme M and a cobalt B substrate analogues. Biochemistry 49, 10922–10921.
Dej, M., Li, X., Zhou, L., and Rag-
dale, S. W. (2009a). Evidence for organometallic intermediates in bac-
terial methane formation involving the nickel cofactor. FEBS Lett. 575, 71–77.
DíMarcos, A. A., Bobik, T. A., and Wolfe, R. S. (1998). Unusual cou-
tryomes of methanogens. Annu. Rev.
Biochem. 59, 353–394.
Drake, H. L., Kond, R., and Mathies, C. (eds). (2006). Actinicyclic Primatean New York. Springer-Verlag.
Duan, E. C., Prakash, D., and Brown, D. (2011). Methyl-coenzyme M reductase from Methanothermocarchae-
us: Methods Enzymol. 494, 305–320.
Duan, E., Cignoret, L., Paikourki, R., Makhat, F. C., DeLey, M., C., Goubeaud, M., Schreiner, G., and
Wondimagegn, T., and Ghosh, A., Finnazzo, C., Harmer, J., Jaun, B., Piskorski, R., Ebner, S., Duin, E. C., Goubeaud, M., Shima, S., Thauer, R. K., and Enders, U. (2008). Comparison of three methyl-coenzyme M reductases from phylogenetically distant organ-
isms reveals unusual amino acid modification, conservation and adaptation. J. Mol. Biol. 380, 329–344.
Gunsolus, R. P., and Wolfe, R. S. (1977). Stimulation of CO2 reduction to methane by methylcoenzyme M in extracts Methanothermanthorhothrix. Biochim. Biochem. Comm. 79, 795–799.
Gunsolus, R. P., and Wolfe, R. S. (1988). Methyl coenzyme M reductase from Methanothermocarchaeus thermophilicus. Arch. Biochem. Biophys. 261, 240–243.
Ha, S. W., Korban, M., Klepech, M., Meier-Kolthoff, W., Meier, D., and Stolzflurny, V. (2007). Interac-
tion of potassium cyanide with the [Ni-4Fe-
5S] active site cluster of CO dehy-
drogenase from carboxyhydrogeno-
hydrogenosulfurans. J. Biol. Chem. 282, 10298–10294.
Harmer, J., Finazzo, C., Paikourki, R., Ebner, S., Dain, E. C., Ghosh, A., et al. (2008). A nickel hydride complex in the active site of methyl-
coenzyme M reductase: implications for the catalytic cycle. J. Am. Chem. Soc. 130, 10972–10979.
Hedman, B., Goubeaud, M., Schreiner, G., and
Zilisch, J. G. (1977). Carbon monox-
ide inhibition by oxygen and diaz-
toia. J. Bacteriol. 132, 118–128.
Hedges, D. J., and Hagen, W . R. (1993). A spec-
troscopic study of the MCr2Red2 form of methyl-
coenzyme M reductase: a pulse EPR and ENDOR study. J. Biol. Chem. 266, 592–593.
Hedges, D. J., Patel, B. K., and Ollier, B. (2000). Taxonomy, phy-
genomics, and ecological diversity of methanogenic Archaea. Annu. Rev. 60, 205–220.
Helmer, J., Voss, A., Wolminkmann, T., and Byegg, H. (2001). Discomulating of F430: quantum chemical perspec-
tives of biological methaneogenesis. Crit. Op. Chem. Biol. 4, 744–750.
Hieber, D. F., and Thauer, R. K. (1997). Purified methanogenic Archaea. Methanobacterium thermoauto-
trophicum, strain Marburg. Nat. Bio-
tech. 15, 769–762.
Hieber, D. F., and Thauer, R. K. (1997). Purified
methanogenic Archaea. Methanobacterium thermoauto-
trophicum, strain Marburg. Nat. Bio-
tech. 15, 769–762.
The nickel enzyme methyl-coenzyme M reductase from methanogenic archaea: in vitro interconversions among the EPR detectable MCR-red1 and MCR-red2 states. J. Biol. Inorg. Chem. 23, 2520–2539.

Richard, R. M., and Rall, D. W. (2018). Ab initio calculations on the thermodynamic properties of arsinepropionate: J. Am. Chem. Soc. 140, 871–879.

Röpstor, S., Borchert, R., Alfreudd, S. P., and Thauer, R. K. (1991a). Methyl-coenzyme M reductase preparations with high specific activity from H2-pretreated cellulos of Methanobac terium ther mautotrophicum: Eur. J. Biochem. 191, 371–375.

Roos, S., Breuning, J., Ma, K., Schwetz, B., Ziemigst, C., Thauer, R. K., et al. (1994b). Methyl-coenzyme M reductase and other enzymes involved in meth anogenesis from O2 and H2 in the extremely thermophile Methanopyrus kandlerii: Arch. Microbiol. 159, 49–55.

Roos, S., and Wolfs, R. S. (1988). Novel biochemistry of methanogens: J. Biol. Chem. 263, 3915–3920.

Schneider, S., Gutenrich, M., Boucher, R., Thauer, R. K., and Jann, B. (2010). The key nickel enzyme of methanogenic catalysts the anabolic oxidation of methane: Nature 465, 606–608.

Scherer, P. A., and Thauer, R. K. (1978). Purification and properties of reduced formylmethyl CO dehydrogenase from Clostridium pasteurianum: a molybdenum iron-sulfur protein: Eur. J. Biochem. 83, 125–135.

Tanner, R. S., Meinemer, M. J., and Nagel, D. P. H. (1989). Formate oxidase of Methanobacterium ther mautotrophicum: Marburg, J. Bacteriol. 171, 6534–6538.

Teelken, C. C., and Ferry, J. G. (1988). Farnesol requirement for electron transport from the carbon monoxide dehydrogenase complex to a molybdenum-hydrogenase in acetate-grown Methanosaeta ther maautotrophicum: J. Biol. Chem. 263, 4075–4079.

Thauer, R. K. (1998). Biochemistry of methanogenesis: a tribute to Mar-jory Stephenson: Microbiology 144, 5277–5286.

Thauer, R. K. (2012). The Wolfe cycle comes full circle: Pro. Natl. Acad. Sci. U.S.A. 109, 15084–15089.

Thauer, R. K., Kastor, A. K., Seedorf, H., Buchholz, W., and Haldenreich, K. (2008). Methanogenic archaea ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6, 579–591.

Wang, M., Tomb, J. B., and Ferry, J. G. (2011). Electron transport in acetate-grown Methanosarcina acetivorans: J. Mol. Biol. 407, 2364–2369.

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