Activation of Methyl-SCoM Reductase to High Specific Activity after Treatment of Whole Cells with Sodium Sulfide†

Donald F. Becker and Stephen W. Ragsdale*

Department of Biochemistry, Beadle Center, University of Nebraska, Lincoln, Nebraska 68588-0664

Received August 28, 1997; Revised Manuscript Received November 17, 1997

ABSTRACT: Here, we report a method to generate the active form of methyl-SCoM reductase (MCR) from *Methanosarcina thermophila*. The protocol involves adding sodium sulfide to a growing cell culture prior to harvest to yield a "ready" (MCR_{ox1}) state of the enzyme. This method can also generate a ready state of the *Methanobacterium thermoautotrophicum* (strain Marburg) MCR. Experiments using sodium ^{35}S-labeled sulfide indicate the ready state that is generated involves a Ni−S adduct. As was shown earlier for the *Mb. thermoautotrophicum* MCR_{ox1} [Goubeaud, M., Schreiner, G. and Thauer, R. K. (1997) *Eur. J. Biochem. 17*, 2374−2377], this ready state is converted to the highly active MCR_{red1} form by reductive activation with Ti(III) citrate. The reduction of MCR_{ox1} to MCR_{red1} with concomitant increase in activity demonstrated that MCR_{red1} is the active form of MCR from *Ms. thermophila*. We also observed the loss of the ^{35}S-sulfide label from the enzyme when MCR_{ox1} was converted to MCR_{red1}. Other states of MCR could be generated in the whole cells by adding different potential ligands to the cell medium; for example, the MCR_{ox2} state was generated by treating cells with sodium sulfite or sodium dithionite.

Methanoarchaea synthesize methane from a variety of sources including CO_2, formate, methanol, methylamines, and acetate (*1*−*5*). The most thermodynamically favorable step in methanogenesis is the reduction of a methyl group to methane by methyl-CoM reductase (*2, 4, 6*). Coenzyme M (HS-CoM), factor F_{430}, and 7-mercaptoheptanoylthreonine phosphate (HS-CoB) are the cofactors utilized by MCR to catalyze the reaction shown in eq 1 (*1, 7*).

$$CH_3\text{-SCoM} + \text{HS-CoB} \rightarrow CH_4 + \text{CoM-S-S-CoB} \quad (\Delta G^{\circ\prime} = -45\ \text{kJ/mol}) \quad (1)$$

MCR has been most extensively characterized from *Methanobacterium thermoautotrophicum* strains Δ*H* and Marburg. These organisms can only use H_2 and CO_2 as their source of electrons and carbon. MCR from these sources has three subunits with a stoichiometry of $\alpha_2\beta_2\gamma_2$ and an apparent molecular mass of 280 kDa determined from both sedimentation and diffusion coefficients (*8*). MCR contains 2 mol (per 280 kDa unit) of tightly but noncovalently bound F_{430}, which is a nickel tetrahydrocorphinoid (*6, 9, 10*). It also has been purified from *Methanosarcina thermophila*, the subject of this study (*11*). This protein was proposed to have an $\alpha\beta\gamma$ form and to contain 1 mol of F_{430} per trimeric unit and K_ms of 3.3 and 59 μM for methyl-SCoM and HS-CoB, respectively (*11*).

F_{430} can assume various oxidation, ligation, and spin states (Scheme 1). EPR spectroscopic studies of *Mb. thermoautotrophicum* (strain Marburg) whole cells have detected at least four states: MCR_{ox1} and MCR_{ox2} that apparently contain Ni in the 3+ oxidation state and MCR_{red1} and MCR_{red2}, in which Ni appears to be in the 1+ oxidation state (*12, 13*). There is also a Ni(II) form that is EPR silent. Whole cell EPR studies have shown that, when cells are bubbled with 100% H_2, MCR is converted to the MCR_{red1} state ($g_z = 2.25$, $g_{x,y} = 2.08$) (*12*). On the other hand, when cells are bubbled with N_2/CO_2 (80%/20%) prior to harvest, the predominant state is MCR_{ox1} ($g_z = 2.23$, $g_{x,y} = 2.16$) (*13*).

Mechanistic studies of MCR have been thwarted by the unavailability of stabile, active enzyme. On the basis of studies with biomimetic models and with partially purified MCR from *Mb. thermoautotrophicum* (strain Marburg), MCR_{red1} [Ni(I) F_{430}] was concluded to be the active state (*12*−*18*). However, purification of the active MCR_{red1} form of the enzyme has not been feasible since this state undergoes facile oxidation to an inactive Ni(II) form (*12, 13*). Although the Ni(II) form of F_{430} and its analogues can be reduced by Ti(III) citrate ($E_m = -0.66$ V, pH 10) to the Ni(I) state at alkaline pH, this one-electron reduction of the Ni(II) state of the enzyme has not been accomplished (*19*). Reductive activation schemes that were described for the *Mb. thermoautotrophicum* Δ*H* and *Ms. thermophila* enzymes yielded specific activities of only 0.1−0.2 units mg^{-1} (*11*, *20*). Therefore, on the basis of the in vivo rate of methanogenesis, even the purified activated enzymes were ∼95−99% inactive. Thus, MCR has been most extensively characterized in the inactive Ni(II) state.

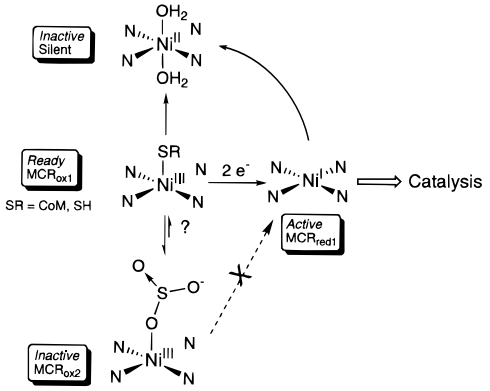
† This work was partially supported by DOE Grant ER20053 (S.W.R.) and a National Research Service Award (1 F32 GM17818-01A1) from NIH to Donald F. Becker.

* Address correspondence to Department of Biochemistry, Beadle Center, University of Nebraska, Lincoln, NE 68588-0664. Phone: 402-472-2943. Fax: 402-472-7842. E-mail: sragsdal@unlinfo.unl.edu.

[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; MCR, methyl-SCoM reductase; Coenzyme M (HS-CoM); 7-mercaptoheptanoylthreonine phosphate (HS-CoB); CHES (2-[*N*-cylcohexylamino]ethanesulfonic acid); MOPS (3-[*N*-morpholino]propanesulfonic acid); BPS, bromopropane sulfonate.

S0006-2960(97)02145-4 CCC: $15.00
Published on Web 02/03/1998

Scheme 1: Proposed Pathway for the Preparation of Ready and Active Forms of MCR from *Ms. Thermophila*



Recently, Thauer et al. (1997) showed that the MCR_{ox1} state of MCR from *Mb. thermoautotrophicum* (strain Marburg) was stable enough to allow purification of the enzyme in this form (*21*). Furthermore, it was demonstrated that the purified MCR_{ox1} could be converted to the MCR_{red1} form by treatment with Ti(III) citrate at pH 9.0. Their pivotal results showed that MCR_{red1} [Ni(I) F_{430}] is an active state of MCR. The MCR_{ox1} state, however, could only be formed in vivo by switching the normal H_2/CO_2 feed gas (80%/20%) to N_2/CO_2 (80%/20%) at 20 min prior to harvest (*21*). This protocol was not applicable to *Ms. thermophila* cells. Thus, to purify and activate MCR_{ox1} from acetate-grown *Ms. thermophila* cells, an alternative method to generate this form of the enzyme needed to be developed.

Here, we describe a method to generate the MCR_{ox1} state in *Ms. thermophila* by adding sodium sulfide to a growing cell culture prior to harvest. We propose that this protocol will allow MCR from any methanogen to be converted to the MCR_{ox1} state and that it will be a valuable tool for mechanistic studies. In addition, we demonstrate the reductive activation of fully purified MCR_{ox1} to MCR_{red1} [Ni(I) F_{430}]. Previously, neither the MCR_{ox1} or the MCR_{red1} states had been observed in *Ms. thermophila*.

MATERIALS AND METHODS

Materials. Ms. thermophila strain TM-1 was a gift from Prof. J. Greg Ferry's laboratory at Pennsylvania State University. *Mb. thermoautotrophicum* (strain Marburg) was obtained from the Oregon Collection of Methanogens, cataloged as OCM82. Methyl-SCoM was prepared from coenzyme M (Sigma) and methyl iodide (Sigma) as described (*22*). After crystallizing methyl-SCoM twice in acetone, its purity was checked by ^{1}H NMR. HS-CoB was prepared as described (*23, 24*). 7-Bromoheptanoic acid was purchased from Karl Industries. *N*-hydroxysuccinamide, *o*-threonine-phosphate and the solvents used in the synthesis were all from Sigma. Bromopropane sulfonate was purchased from Sigma. The purity of HS-CoB was ascertained by ^{1}H NMR.

A stock solution of 200 mM Ti(III) citrate was prepared by dissolving 0.75 g of Ti(III) trichloride (Fluka) in 25 mL of 250 mM sodium citrate (Sigma) under anaerobic conditions and then adjusting the pH to 7.0 with sodium bicarbonate (Sigma). Natural abundance and sodium ^{35}S-labeled sulfide were purchased from Sigma and Amersham, respectively. All buffers, media ingredients, and other reagents were acquired from Sigma. Solutions were prepared using nanopure deionized water. N_2 (99.98%), N_2/H_2 (90%/10%), Ar (99.8%) and CH_4/N_2 (0.2%/99.8%) were obtained from Linweld (Lincoln, NE).

Ms. thermophila Growth and Harvest Conditions. Ms. thermophila strain TM-1 was cultured on acetate at 50 °C and pH 6.8 in a 5-L New Brunswick fermentor with a pH auxostat (*25*). The media was prepared as previously described (*25*). In the late log phase, 500 mL of supplemental media was added to the culture. The supplemental media (500 mL) contained 18.4 g of ammonium chloride, 2.5 g of yeast extract, 2.5 g of tryptone, 2.8 g of monobasic potassium phosphate, 2.18 g of dibasic potassium phosphate, 20 mg of nickel chloride, 0.158 g of calcium chloride, 57 mg of ferrous ammonium sulfate, 0.22 g of magnesium sulfate, 2.26 g of sodium chloride, and 1.5% (vol/vol) vitamin and 10% (vol/vol) trace element solutions which were previously described (*26*). *Ms. thermophila* was harvested at the end of log phase under anaerobic conditions by continuously decanting the medium above the settled cells. *Ms. thermophila* was cultured on H_2/CO_2 according to the protocol described earlier in which the organism was first grown in nitrogen limiting media (0.5 mM ammonium chloride) containing acetate and then switched to H_2/CO_2 in the absence of acetate (*26*−*28*).

Mb. thermoautotrophicum (strain Marburg) was cultured on $H_2/CO_2/H_2S$ (80%/20%/0.1%) at 65 °C in a 14-L New Brunswick fermentor (*21*, *29*). Media was prepared as previously described (*29*).

Treatment of Ms. thermophila Cells with Sulfide and Sulfite. The 5-L *Ms. thermophila* culture was reduced to 2-L, sodium sulfide or sodium sulfite was added, and the culture was incubated in an oven at 50 °C for 1 h to induce the MCR_{ox1} state. Longer incubation times (e.g., 2 h) did not increase the MCR_{ox1} EPR signal. The shortest incubation time needed to form the MCR_{ox1} signal by sodium sulfide was not determined; however, it is possible that shorter times could be sufficient since 20 min of incubation with sodium sulfite at 50 °C was sufficient to generate the MCR_{ox2} form.

Sodium ^{35}S-Labeled Sulfide Treatment of Ms. thermophila and Mb. thermoautotrophicum (Strain Marburg). When *Ms. thermophila* was treated with 20 mM sodium ^{35}S-labeled sulfide, only 0.5 L of culture was used due to the cost of the sodium ^{35}S-labeled sodium sulfide and also to minimize the amount of radioactive material spent. For the treatment of *Mb. thermoautotrophicum* (strain Marburg) with 20 mM ^{35}S-labeled sulfide, a 10-L culture ($A_{578} \sim 3.0$) was first concentrated to 0.4 L and then gassed for 1 h with $H_2/CO_2/H_2S$ (80%/20%/0.1%) at 65 °C before adding sodium ^{35}S-labeled sulfide to the culture. After 20 min at 65 °C, the 0.4 L culture was then cooled on ice. The radioactivity of purified MCR from both *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg) was determined by suspending the sample in 18 mL of Ecolite (ICN Biomedicals) and counting in a Packard 1600TR scintillation analyzer. The filtrate from the concentration step was used as a background for the scintillation counting. The Penefsky column (5 mL) was made by equilibrating Sephadex G-50 (Sigma) with 50 mM Tris-HCl (pH 7.6) and removing excess buffer by a clinical centrifuge (*30*). MCR samples were

also passed through the column by spinning in the clinical centrifuge.

Purification of MCR_{ox1} from Ms. thermophila. The purification and manipulations of MCR were performed under a N_2/H_2 (95%/5%) atmosphere in an anaerobic chamber (Coy Instruments). All steps of the purification were performed at 4 °C. After treating cells with sodium sulfide, 50−70 g of cells (wet mass) were immediately suspended in 0.5 M potassium phosphate (pH 7.6) containing 4 mM Ti(III) citrate, 10 mM methyl-SCoM, and 10 mM methyl viologen. Lysis of the cell suspension was achieved with an Ultrasonic Liquid Processor (Misonix, Inc) programmed for a total sonication time of 8 min (20 s pulses and 45 s pauses). The cell lysate was then ultracentrifuged at 30 000 rpm for 30 min in a Type 35 rotor (Beckman). Fractional ammonium sulfate precipitation (60 and 100%) was then used to purify MCR (*13*). After MCR was precipitated with 100% ammonium sulfate, the pellet was resuspended in 150 mL of 50 mM potassium phosphate (pH 6.8) containing 10 mM methyl-SCoM and 10% glycerol. The supernatant was then loaded at 90 mL/h on a high-performance Q-Sepharose (Sigma) column (2.5 cm × 20 cm) equilibrated with 50 mM potassium phosphate (pH 6.8) containing 10% glycerol. MCR was eluted by applying an 800 mL linear gradient (90 mL/h) from 0.2 to 0.4 M sodium chloride in 50 mM potassium phosphate (pH 6.8) containing 10% glycerol. Fractions containing the highest activity were then pooled and concentrated under argon (that had been passed through an oxisorb column) pressure in either a 10-mL omegacell (Filtron) or a 50-mL Amicon ultrafiltration cell with a 30 kDa molecular mass cutoff. This scheme routinely yielded 60 mg of purified MCR.

MCR from *Mb. thermoautotrophicum* (strain Marburg) was purified as described (*21*).

Protein Determination. Protein was determined by the Bradford method using the Bio-Rad reagent (Bio-Rad) with cytochrome *c* as a standard (*31*). Protein quantitation by this assay using cytochrome *c* agreed with the protein quantitation determined by amino acid analysis of purified MCR. Cytochrome *c* concentrations were measured spectrophotometrically using an extinction coefficient of 105 000 cm^{-1} M^{-1} at 410 nm (*32*). F_{430} content was estimated by using the extinction coefficient of 22 000 cm^{-1} M^{-1} at 420 nm (*33*).

Measurement of MCR Activity. MCR assays were performed at 60 °C in rubber-sealed 8-mL serum vials. The standard assay mixture contained 20 mM methyl-SCoM, 1.2 mM HS-CoB, 0.6 mM aquocobalamin, 25 mM Ti(III) citrate, and 500 mM MOPS (pH 7.0) in a final volume of 0.4 mL. It was earlier reported that ATP stimulated MCR activity; however, we observed no enhancement of MCR activity when ATP was included in the assay. The reaction was started by increasing the temperature from 4 to 60 °C. Methane generated by MCR during the assay was determined by withdrawing gas samples at specific time points for analysis by gas chromatography (Varian model 3700 equipped with an Alteck heliflex AT-Q column and flame ionization detector). Rates of methane formation were then calculated from the linear time course. One unit of MCR activity is equal to 1 μmol of methane min^{-1}.

MCR Activation. Ms. thermophila MCR was activated by incubating the enzyme (5−50 μg) at 45 °C for 30 min in a solution (0.2 mL total volume) containing 200 mM CHES (pH 9.0), 20 mM Ti(III) citrate, and 20 mM methyl-SCoM. Then the activation mixture was cooled to 4 °C and 0.14 mL of 2 M MOPS (pH 6.5), 16 μL of 30 mM HS-CoB, 16 μL of 15 mM aquocobalamin, and 30 μL of 200 mM Ti(III) citrate were added. The final pH was 6.9−7.0. The MCR assay was then performed as described above. For the sodium ^{35}S-labeled sulfide experiments, *Mb. thermoautotrophicum* (strain Marburg) MCR was activated by heating at 60 °C for 30 min in the presence of 1 mM Ti(III) citrate and 10 mM methyl-SCoM at pH 9.0 (*21*). The specific activity of MCR from *Mb. thermoautotrophicum* (strain Marburg) after activation was 10 units mg^{-1}.

Ti(III) citrate has strong EPR and UV−visible spectra that interfere with those of MCR. Therefore, to correlate activity with spectroscopic changes, MCR was activated in a sealed UV−visible cuvette or EPR tube in a mixture containing 800 mM CHES (pH 9.0), 20 mM methyl-SCoM and 0.5 mM Ti(III) citrate. Aliquots were removed from the activation mixture into a standard MCR activity assay.

Spectroscopy of MCR. UV−visible spectra of MCR were recorded with a diode array spectrophotometer (Beckman Series 7000). EPR spectra were recorded on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller, a Hewlett-Packard Model 5340 automatic frequency counter and Bruker gaussmeter. The EPR spectroscopic parameters are given in the figure legends. Double integrations of the EPR spectra were performed with copper perchlorate (1mM) as the standard.

Data Manipulations and Calculations. UV−visible spectral subtractions of MCR were performed with Spectra Calc (Galatic) and Sigma Plot software programs. Subtractions of the Ni(II) state from the MCR spectrum were based on the EPR quantitation of the MCR_{ox1} signal. To determine the Ti(III) citrate required for 50% activation of MCR, the data were fit to an equation in which Ti(III) was treated as an essential activator as described (*34*). For the active-site titration of MCR with bromopropane sulfonate, the data were fit to the linear equation (eq 2) for simple competitive inhibition (*35*), where [I], v_I, v_o, and K_I are defined as the inhibitor concentration, velocity in the presence of inhibitor, velocity in inhibitor's absence, and inhibition constant, respectively.

$$[I]t/(1 - v_I/v_o) = K_I(1 + [S]/K_m)v_o/v_I + [E]_t. \quad (2)$$

RESULTS

Generation of the MCR_{ox1} state in vivo. Initially, variable amounts (from 0 to 0.15 spins/mol) of MCR_{ox1} were observed in purified MCR from *Ms. thermophila*. Furthermore, no basis for the formation of the MCR_{ox1} state could be identified. We typically observed a mixture of MCR_{red1} (g_z = 2.25, $g_{x,y}$ = 2.07) and MCR_{ox1} (g_z = 2.23, $g_{x,y}$ = 2.17). Attempts to induce MCR_{ox1} formation by gassing the culture with N_2/CO_2 using the protocol described for *Mb. thermoautotrophicum* (strain Marburg) were unsuccessful (*21*). Since we assumed that this state represented the oxidized enzyme, various modifications were attempted. These included altering the time of bubbling with CO_2, washing and resuspending the cell pellet in fresh medium lacking acetate (to ensure the absence of an electron donor) or in potassium

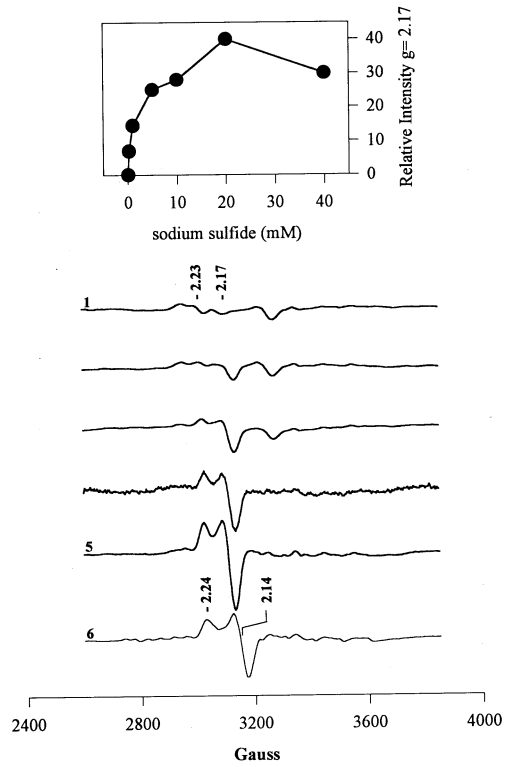


FIGURE 1: Generation of the MCR_{ox1} state. (Lower panels) *M. thermophila* cells were treated with different concentrations of sodium sulfide (curves 1−5, 0.2, 1, 5, and 20 mM sodium sulfide, respectively) or 20 mM sodium sulfite (curve 6) and studied by whole cell EPR spectroscopy. (Upper panel). The EPR signal intensity at $g = 2.17$ was plotted versus sodium sulfide concentration in the cell medium. Experimental conditions: temperature, 120 K; microwave power, 10 mW; microwave frequency, 9.43 GHz; receiver gain, 2×10^4; modulation amplitude, 12.8 G; modulation frequency 100 kHz.

phosphate buffer. None of these modifications were successful in generating the MCR_{ox1} state of the enzyme. We considered that this may have been because *Mb. thermoautotrophicum* (strain Marburg) was grown autotrophically on H_2/CO_2, while *Ms. thermophila* was cultured on acetate. However, when we cultured *Ms. thermophila* on H_2/CO_2, as with the acetate-grown cells, only a low amount of the MCR_{ox1} EPR signal was observed in the whole cells after treatment with N_2/CO_2.

A different approach was then initiated which was based on the premise that, by controlling the Ni ligation state, the activity of MCR could also be controlled. This involved a series of studies that tested the effects of adding different chemical agents to the culture medium prior to harvest. When sodium sulfide was added, a significant amount of the MCR_{ox1} state was elicited (Figure 1). As the sulfide concentration was increased to 20 mM, the MCR_{red1} state disappeared as the MCR_{ox1} became dominant. The methanogenesis activity decreased with increasing amounts of sodium sulfide. A 60% inhibition of methanogenesis was observed in cultures containing ∼5 mM sodium sulfide. This is the same concentration at which significant amounts of the MCR_{ox1} form began to appear. Previous work has also reported inhibition of methanogenesis at ∼2−5 mM sodium sulfide (*36*).

Increasing the sodium sulfide concentration to 20 mM resulted in an increase in pH of the culture media from 7.0 to 8.8. However, high pH was not responsible for generating the MCR_{ox1} state, since, when the pH of the culture media was increased to pH 8.8 with CHES buffer, but without sodium sulfide addition, the amount of MCR_{ox1} in the whole cells did not increase after 1 h of incubation at 50 °C. In addition, when potassium phosphate buffer was added (final concentration, 250 mM) with sodium sulfide (20 mM, final) to keep the pH constant at 7.2, the amount of MCR_{ox1} was similar to that observed at pH 8.8 (data not shown). Thus, generation of the MCR_{ox1} state in *Ms. thermophila* whole cells depends on sulfide addition and not pH changes.

Adding sodium sulfite to the culture medium of *Ms. thermophila* prior to harvest (final pH 6.5) elicited the formation of a different species of MCR, marked by an EPR signal (Figure 1, curve 6) with a morphology and g values ($g_z = 2.24$, $g_{x,y} = 2.14$) similar to those observed in the MCR_{ox2} state of *Mb. thermoautotrophicum* (strain Marburg) (*12*). Thus, sodium sulfide and sodium sulfite appear to enter the cells and stabilize specific states of MCR.

Treatment of *Mb. thermoautotrophicum* (strain Marburg) cells with sodium sulfide or sodium sulfite for 20 min before harvest also resulted in formation of the MCR_{ox1} and MCR_{ox2} species, respectively. Gassing the culture with N_2/CO_2 was not required. The EPR spectra of the MCR_{ox1} and MCR_{ox2} states from the two methanogens are indistinguishable. Therefore, we propose that this method of generating the "MCR_{ox}" states by adding sulfide and sulfite will be useful with other methanogens to generate MCR_{ox1} and MCR_{ox2}.

Purification and Stability of MCR_{ox1} from Ms. thermophila. MCR was purified to >90% homogeneity as determined by densitometry of the SDS−polyacrylamide gel of the sample after the ion exchange step. The MCR_{ox1} EPR signal ($g_z = 2.23$, $g_{x,y} = 2.17$) of the purified *Ms. thermophila* enzyme quantitated to 0.4−0.5 spin/mol of MCR. Since no other EPR signals were observed, the remaining MCR must be present in the Ni(II) form. The UV−visible spectrum of purified MCR (Figure 2) contained an absorbance maximum at 423 nm and a shoulder at 390 nm. The Ni(II) form of MCR absorbs maximally at 421 nm (Figure 2, inset) with a shoulder at 440 nm. Since the spectrum in Figure 2 contains a mixture of MCR_{ox1} and the Ni(II) state, spectral deconvolution was performed to obtain a clean absorption spectrum of the MCR_{ox1} state (Figure 2, inset). The maximum absorbance of MCR_{ox1} occurs at 425 nm with a shoulder at 390 nm.

The stability of purified MCR_{ox1} from *Ms. thermophila* was determined by storing the protein aerobically and anaerobically at 4 °C and removing samples at various times for EPR spectroscopic measurements. Both samples showed an initial decrease in the MCR_{ox1} signal (∼50% decay in the first 24 h) similar to that reported for MCR_{ox1} from *Mb. thermoautotrophicum* (strain Marburg) (*21*). By the second day, however, the rate of decay of the MCR_{ox1} state was much slower with the sample stored in air decaying faster (∼6%/day) than the anaerobically stored sample (∼3%/day). Thus, oxygen appears to increase the MCR_{ox1} decay rate in this second slower phase. Because of the initial rapid decay of the MCR_{ox1} state to the inactive Ni(II) state, experiments were performed within the first 24 h after purification.

Activation of MCR. Reduction of *Ms. thermophila* MCR_{ox1} was achieved using a method similar to that reported for MCR_{ox1} from *Mb. thermoautotrophicum* (strain Marburg)

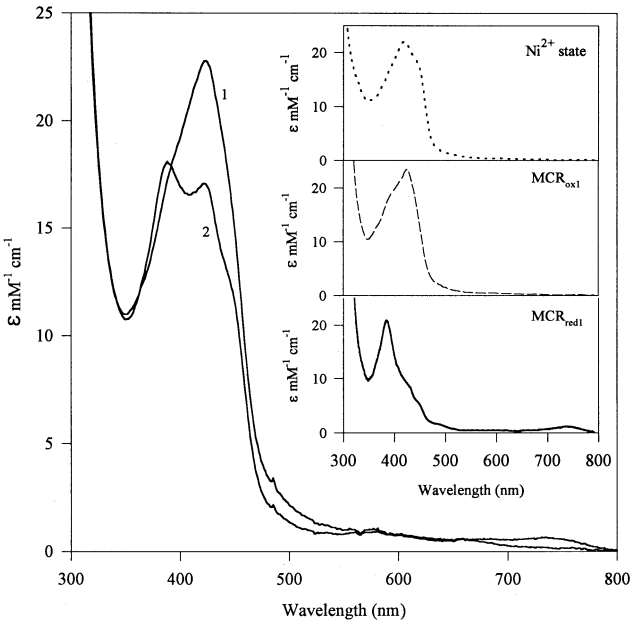


FIGURE 2: UV−visible spectra of the MCR_{ox1}, MCR_{red1}, and Ni-(II) states. (1) Purified MCR (19 μM) containing 0.5 spin of the MCR_{ox1} EPR signal per mole of MCR. (2) Reduction of 1 with Ti(III) citrate at 45 °C for 30 min. (Inset) (•••) Ni(II) state of MCR (12 μM); (---) MCR_{ox1} spectrum generated by subtracting the spectrum of the Ni(II) state of MCR (9 μM) from the composite spectrum (1); (−) MCR_{red1} (7.5 μM) spectrum generated by subtracting the spectrum of the Ni(II) state (9 μM) and the spectrum of MCR_{ox1} (2.5 μM) from the composite reduced spectrum (2).

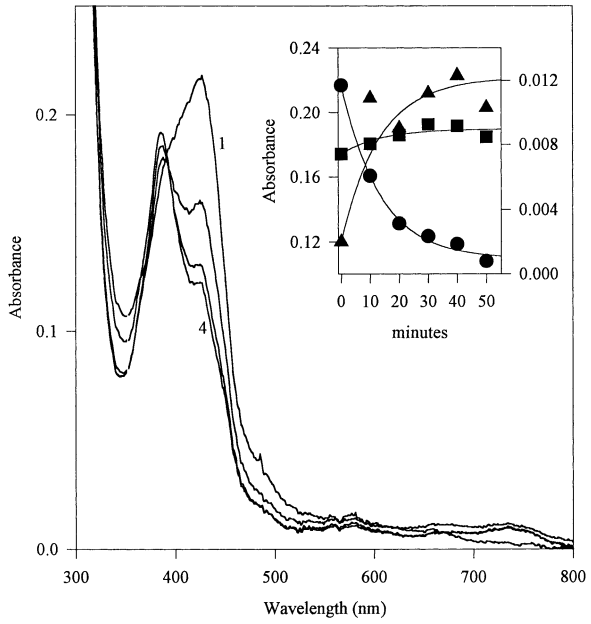


FIGURE 3: Kinetics of the MCR_{ox1} reduction. MCR (19 μM) was incubated at 45 °C with 0.5 mM Ti(III) citrate for 0−50 min (curves 1−4, 0 min, 10 min, 20 min, 30 min). The Ni(I) state of MCR (9 μM) was subtracted from the spectra. INSET: The plot of the absorbance at 388 nm (■), 425 nm (●), and 750 nm (▲) during the reduction of MCR_{ox1} with Ti(III) citrate at 45 °C. A rate constant of $k = 0.08 \pm 0.01$ min^{-1} was determined for the MCR_{ox1} decay at 425 nm by a fit to a theoretical single-exponential equation. The increases in absorbance at 388 and 750 nm showing the formation of MCR_{red1} are fit with single-exponential equations using the rate constant determined from the decay at 425 nm.

(*21*). First, different pH (pH 7.8−10.0) and temperature (25 − 60 °C) ranges were tested to optimize the amount of MCR_{red1}, and the activation was followed by UV−visible spectroscopy. It was observed that, at high pH (pH > 9.0) and at temperatures above 50 °C, MCR denatured and released the cofactor, Ni(II) F_{430}. At 45 °C and pH values between 8.5 and 9.0, the maximum amount of MCR_{red1} was formed. Very little MCR_{red1} could be generated at 25 °C at the optimum pH. Activation of MCR_{ox1} from *Mb. thermoautotrophicum* (strain Marburg) is optimal at 60 °C and pH ≥ 9.0 (*21*).

Next, the Ti(III) citrate concentrations used for the reductive activation were varied to determine the amount of Ti(III) citrate required for optimal MCR activity. Methyl-SCoM was added to the activation mixture since it has been reported to stabilize the MCR_{red1}. After a sharp dependence up to 2.5 mM Ti(III) citrate, only a small increase in the MCR activity was observed upon increasing the Ti(III) citrate concentration further to 20 mM. Fifty percent activation was estimated to occur at 1.4 ± 0.4 mM Ti(III) citrate (data not shown).

The specific activity of the purified protein before activation was 0.24 units mg^{-1}. This activity is the same as that reported previously for purified MCR from *Ms. thermophila* (*11*). The specific activity of MCR after activation was routinely 5−7 units mg^{-1}. After concentrating MCR, the specific activity usually decreased to 4−5 units mg^{-1}. The rate of methane synthesis in growing *Ms. thermophila* cells (wet mass of 57 g, resulting in ~800 mg total soluble protein) is 0.059 mol of methane h^{-1} (*25*). Since MCR amounts to ~10% of cell protein, the expected specific activity is estimated to be 10−15 units mg^{-1} (*8*).

To follow the activation spectroscopically, the concentration of Ti(III) citrate was decreased. Reduction of MCR with 0.5 mM Ti(III) citrate (pH 9.0, 45 °C) (Figure 2) results in formation of MCR_{red1} [Ni(I) F_{430}] with increases in absorbance at 388 and 750 nm as described earlier (*19*, *21*). Figure 2 (inset) shows the MCR_{red1} spectrum after subtracting the contributions of the residual Ni(II) and MCR_{ox1} species. This spectral deconvolution allowed the kinetics of MCR_{red1} formation to be assessed (Figure 3). MCR_{red1} formation was nearly complete in 30 min ($k = 0.08$ min^{-1}).

The reduction of MCR with 0.5 mM Ti(III) citrate was also monitored by EPR spectroscopy (Figure 4). The inset to Figure 4 shows the conversion of MCR_{ox1} to MCR_{red1} at selected time points. The rate of reduction was calculated to be similar to that measured by UV−visible spectroscopy, with approximately 60% of the MCR_{ox1} (17.5 μM) converted to the MCR_{red1} (or 10.5 μM MCR_{red1} corresponding to 0.3 mol of MCR_{red1}/mol of MCR) state in 30 min. Incomplete reduction was also observed with MCR_{ox1} from *Mb. thermoautotrophicum* (strain Marburg) with 0.5 mM Ti(III) citrate (*21*). The rate constants for the decay of MCR_{ox1} and formation of MCR_{red1} were similar to those obtained by UV−visible spectroscopy (Figure 4). These data strongly suggest that MCR_{ox1} is the species converted to MCR_{red1}.

The specific activity of MCR increased as the amount of MCR_{red1} increased (Figure 5), strongly supporting the conclusion that MCR_{red1} is the active form of MCR (*21*). The results of a typical assay are shown in the inset to Figure 5.

Efficacy of the sulfide treatment was assessed by performing an active-site titration of MCR using the potent inhibitor,

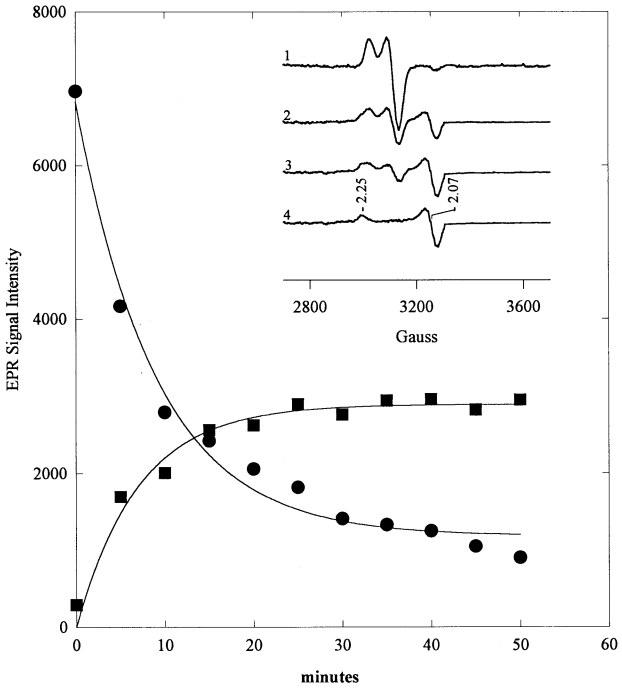


FIGURE 4: Reductive activation of purified MCR. MCR (35 μM) was incubated at 45 °C with 0.5 mM Ti(III) citrate for 0−50 min and measured by EPR spectroscopy at each time point. The relative EPR intensities of MCR_{ox1} (●) and MCR_{red1} (■) during the reduction of MCR with Ti(III) citrate were fit to a theoretical single-exponential decay and increase, respectively. The rate constants determined were $k = 0.11 \pm 0.01$ min^{-1} for the MCR_{ox1} decay and $k = 0.11 \pm 0.01$ min^{-1} for the MCR_{red1} formation. (Inset) EPR spectra of MCR (35 μM) at various times during the reduction (curves 1−3; 0, 10, 35 min, respectively). (Curve 4) MCR_{red1} EPR spectrum generated by subtracting residual MCR_{ox1} from curve 3. Experimental conditions: temperature, 80 K; microwave power, 10 mW; microwave frequency, 9.43 GHz; receiver gain, 2×10^4; modulation amplitude, 12.8 G; modulation frequency 100 kHz.

BPS (Figure 6) (*37, 38*). A sample containing MCR_{ox1} (0.5 spin/mol) was activated [20 mM Ti(III) citrate at 45 °C for 30 min] and then treated with varying concentrations of the active-site inhibitor. The number of active-sites per mole of MCR (0.5 ±0.2) was equivalent to the amount of MCR_{ox1} providing further evidence that the active form of MCR is derived from the MCR_{ox1} state. An inhibition constant of 0.08 ± 0.01 μM was determined for BPS with activated MCR_{ox1}.

Mechanism of the MCR_{ox1} Generation by Sodium Sulfide. To determine if the effects of sulfide addition could arise from the formation of a Ni−S adduct, both *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg) cells were treated with 20 mM sodium ^{35}S-labeled sulfide and MCR was subsequently purified (see Materials and Methods). The MCR fractions from the mono-Q column were pooled, concentrated, and analyzed by EPR spectroscopy and for ^{35}S-sulfide content by scintillation counting. In three different preparations, the amount of MCR_{ox1} as determined by EPR spectroscopy correlated with the amount of ^{35}S-labeled sulfide (Figure 7), indicating that sulfide enters *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg) cells, binds to the Ni site in F_{430} and remains bound during the purification procedure.

The sulfide adduct was shown to be released from MCR during the reductive activation of MCR_{ox1} to MCR_{red1}. Two samples of MCR from *Mb. thermoautotrophicum* (strain

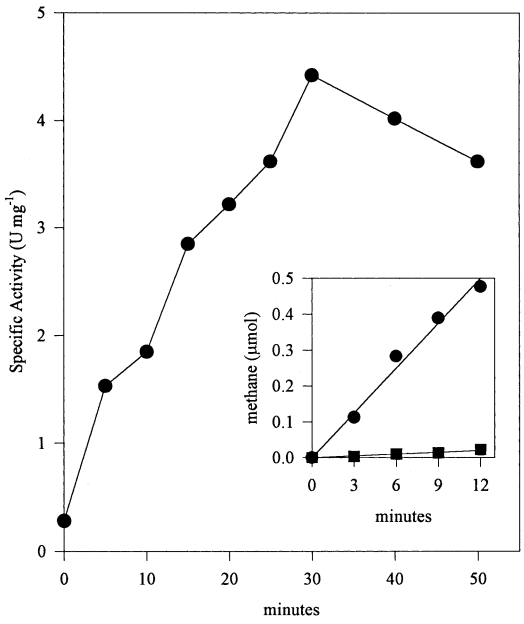


FIGURE 5: Time course of the activation of MCR. MCR was incubated (pH 9.0) with 0.5 mM Ti(III) citrate and 20 mM methyl-SCoM at 45 °C for 0−50 min and assayed. (Inset) Results of a typical assay of MCR activity (8 μg) before (■) and after (●) activation. The calculated specific activity of this enzyme sample was 5 units mg^{-1}.

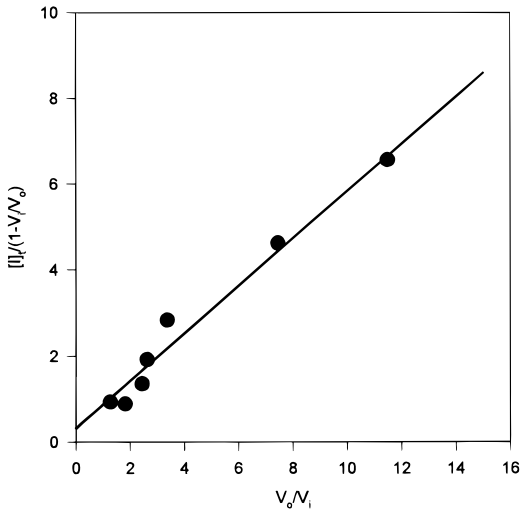


FIGURE 6: Active-site titration of activated MCR (0.67 μM) with BPS. A preparation with 0.5 spin of MCR_{ox1} per mole of MCR was activated with 20 mM Ti^{3+} citrate at 45 °C for 30 min and titrated with the active-site inhibitor. The data are fit to a linear equation for simple competitive inhibition with the intersection at the ordinate representing the amount of active-sites present. This treatment of the data estimated 0.5 ± 0.2 active sites/mole of MCR. An inhibition constant of 0.08 ± 0.01 μM was determined for BPS with activated MCR_{ox1}.

Marburg) containing 0.8 spin of MCR_{ox1} and 0.64 mol of ^{35}S-labeled sulfide/mol of MCR were activated. The samples were then applied to a Penefsky-type gel filtration column, and the amount of remaining ^{35}S-label was determined. After activation, the amount of MCR_{ox1} and MCR_{red1} present was 0.05 and 0.65 spin/mol of MCR, respectively, indicating that conversion of the MCR_{ox1} to the MCR_{red1} is nearly quantitative. After gel filtration, the amount of ^{35}S-labeled sulfide had decreased to 0.10−0.13 mole/mol of MCR in both samples. The amount of ^{35}S-sulfide bound to MCR in a control sample (with 0.69 mol of ^{35}S-sulfide/mol of MCR)

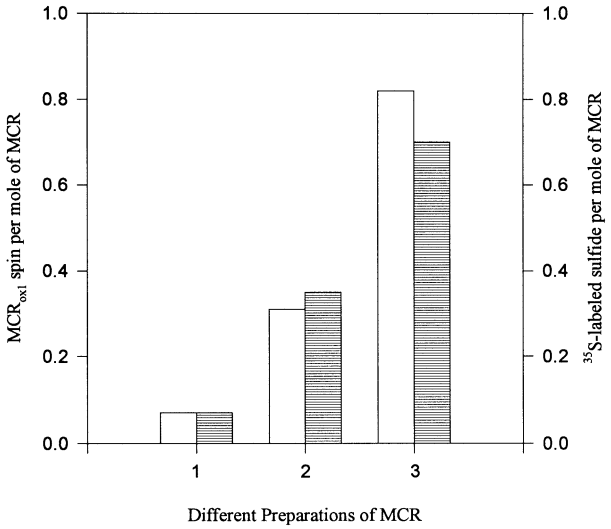


FIGURE 7: Correlation of the amount of MCR_{ox1} (open bars) and ^{35}S-label (striped bars) in MCR purified from *Ms. thermophila* (preparation 2) and *Mb. thermoautotrophicum* (strain Marburg) (preparations 1 and 3). For each preparation of MCR the amount of MCR_{ox1} was quantitated by EPR spectroscopy and the amount of ^{35}S-label was determined by scintillation counting. EPR experimental conditions: temperature, 120 K; microwave power, 1.26 mW; microwave frequency, 9.43 GHz; receiver gain, 2×10^4; modulation amplitude, 12.8 G; modulation frequency 100 kHz.

that was not activated did not decrease after passage through a Penefsky desalting column. Therefore, activation of MCR appears to displace the sulfide (e.g., from the Ni) which can then be removed from MCR by gel filtration. This result is consistent with earlier studies of Ni model compounds in which it has been shown that Ni(I) F_{430} prefers a four-coordinate square planar geometry (*39*, *40*).

DISCUSSION

The most acute and persistent problem in studying the catalytic mechanism of methane formation by MCR has been the enzyme's low activity. It has seemed fruitless to consider mechanistic studies on an enzyme with less than 1% activity. Our efforts to overcome this problem have been guided by two important conclusions of the work of Goubeaud et al. (1997): (i) the MCR_{ox1} state was stable enough to be purified, and (ii) MCR_{ox1} could be reduced in vitro to the active MCR_{red1} state (*21*). Thus, our objective was to find a way to optimize the amount of MCR_{ox1} from *Ms. thermophila*. Unfortunately, the protocol of changing the feed gas from H_2/CO_2 to N_2/CO_2 before harvesting the cells could not be extended to *Ms. thermophila*. Therefore, it was necessary to develop an alternative protocol that was useful for *Ms. thermophila* and generally applicable.

We conceived that one possible strategy was to induce the MCR_{ox1} form by controlling the Ni ligation state of F_{430} in MCR. It had been suggested that ligand exchange at the sixth axial position of Ni in F_{430} is enhanced if a strongly coordinating fifth axial ligand is present (*41*). Resonance Raman studies of model compounds also demonstrated that subtle changes in the structure of the nickel tetrapyrrolic macrocycle can substantially alter ligand affinity and reactivity (*42*). Thus, we decided to treat *Ms. thermophila* with different ligands that could plausibly enter the cells and coordinate to the Ni in MCR, but still be labile enough to be displaced during activation or catalytic turnover. Sodium sulfide and sodium sulfite were selected since they could mimic the two "faces" of CoM by providing two different liganding interactions with Ni, a sulfur and an oxygen, respectively. Sodium sulfide and sulfite have also been shown to readily enter growing cells (e.g., *Mb. thermoautotrophicum* strain Δ*H*) (*43*). Although this strategy seemed plausible, we were surprised to observe the MCR_{ox1} and MCR_{ox2} EPR signals when whole cells were treated with sodium sulfide and sodium sulfite, respectively. A high proportion of the enzyme was converted to the MCR_{ox1} form in both *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg), ranging 0.4−0.5 and 0.6−0.8 spin/mol of MCR, respectively. The correlation between the amount of ^{35}S-label and MCR_{ox1} present in the purified MCRs of *Ms. thermophila* and *Mb. thermoautotrophicum* cells strongly suggests that sulfide ligates to the Ni site in F_{430}. Some other possible causes for the formation of the MCR_{ox} states besides the ligation of sulfide or sulfite to the Ni in F_{430} MCR were ruled out. For example, it is clear that changes in pH during the treatment of the whole cells are not responsible for the stabilization of MCR_{ox1} and MCR_{ox2}. Furthermore, changes that occur in the redox potential of the cell medium when sulfide or sulfite are added are unlikely to play a role since MCR_{ox2} can also be formed by treating whole cells with sodium dithionite (data not shown). Measurement of the redox potential of the growth cell medium poised by 20 mM sodium dithionite showed that it was 0.23 V more negative than that poised by 20 mM sodium sulfite in the cell medium. Thus, MCR_{ox2} can be stabilized at two drastically different cell culture redox potentials. These observations suggests that the changes that occur in the redox potential of the cell medium are not solely responsible for the formation of MCR_{ox2}, a result that may also pertain to MCR_{ox1}.

The generation of MCR_{ox1} by sodium sulfide treatment of growing cells was also successful with H_2/CO_2-grown *Mb. thermoautotrophicum*. We project that it will be applicable to other methanogens. Interestingly, the addition of sulfide to cell extracts or purified enzyme did not generate the MCR_{ox1} state, indicating the existence of an enzymatic process that promotes Ni−S formation in vivo. Presumably this is a labile system that is disrupted upon cell lysis.

Our results demonstrate that the MCR_{ox1} state of the enzyme that is generated by sulfide addition to *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg) growing cells can be nearly quantitatively converted to the MCR_{red1} state. We propose the "MCR_{ox1} state" can be referred to as a "ready state" (Scheme 1) in analogy to the terminology used in classifying the different forms of the Ni-containing hydrogenase (*44*). This terminology was also used earlier in describing the activation of MCR (*11*).

It will be interesting to know if the MCR_{ox1} state generated by sulfide treatment of *Ms. thermophila* and *Mb. thermoautotrophicum* (strain Marburg) is analogous to the MCR_{ox1} state formed in *Mb. thermoautotrophicum* by replacing the feed gas with N_2/CO_2 (*21*). EPR spectra are highly sensitive to the environment, including the ligation state. The similar EPR spectra and activation properties of MCR obtained by the two protocols suggests that the MCR_{ox1} state may be a Ni-thiol adduct in both cases. For the MCR_{ox1} from *Mb. thermoautotrophicum*, this mercapto-ligand may be sulfide (the cells are grown with ∼ 0.1% H_2S in the medium) or

tightly bound HS-CoM.

Scheme 1 is a proposed activation mechanism for MCR from *Ms. thermophila*. MCR_{ox1} is defined as a ready state and MCR_{red1} as an active state. This scheme is similar to an activation mechanism proposed earlier that included "inactive" Ni(II), "ready", and "active" MCR states (*11*). We have formulated the ready state as Ni(III) because conversion of MCR_{ox1} to MCR_{red1} requires reduction, although it is still not conclusively known whether MCR_{ox1} contains Ni(I) or Ni(III) (*21*). Ti(III) citrate could function by either reducing Ni(III) to Ni(I) or by reducing a ligand of a Ni(I) species (*21*). Thus, conversion of MCR_{ox1} to MCR_{red1} is proposed to involve a two electron reduction and displacement of the axial ligand to form a four-coordinate Ni(I) state. On the basis of EPR and ENDOR studies of the Ni(I) form of uncomplexed F_{430}, it was concluded that there are no axial ligands in this redox state (*40*). One issue is why only the MCR_{ox1} state can undergo reductive activation. The results from the experiments with ^{35}S-labeled sulfide suggest that the sulfide ligand can be displaced during activation allowing formation of the four-coordinate Ni(I) state. We surmise that the Ni active site is inaccessible to substrate (or BPS) and reductants unless an appropriate ligand is present that is stable enough to survive purification, promote reductive activation of the MCR_{ox1} state to MCR_{red1}, and dissociate from the activated enzyme to allow substrate binding. Activation may require a conformational movement of the protein. This may partially explain the requirement for heat near the physiological growth temperature during the reductive activation. It was suggested earlier that subtle changes in the F_{430} environment may be necessary for activation (*45*). Synthetic model studies have also previously suggested that the ability of F_{430} to change conformation facilitates Ni reduction (*46*).

It was earlier proposed that Ti(III) citrate or ferredoxin coupled with ATP hydrolysis was required for the conversion of the MCR inactive state to the MCR active state. This scheme was based on the reactivation of inactive MCR to specific activities of <0.1 unit mg^{-1}. Here, we have identified a ready state (MCR_{ox1}) and an active state (MCR_{red1}) and have also shown that ATP and ferredoxin are not required for activation. Once the Ni(I) state is formed, it can enter the catalytic cycle by reacting with methyl-CoM. An available axial coordination site at the Ni(I) of F_{430} would allow the formation of a methyl-Ni species, which has been proposed to be an intermediate in the catalytic cycle and has also been detected by ^2H NMR spectroscopy of a F_{430} pentamethyl ester after methylation of the F_{430} derivative (*14*, *47*).

The MCR_{ox2} state is also included in Scheme 1. The species generated by treating *Ms. thermophila* cells with sodium sulfite or dithionite has spectroscopic properties that are similar to those of the MCR_{ox2} state described earlier for the *Mb. thermoautotrophicum* (strain Marburg) MCR by Albracht et al. (*12*). In their experiments, MCR_{ox2} was generated by gassing the *Mb. thermoautotrophicum* (strain Marburg) cells with air. No spectroscopic characterization of purified MCR_{ox2}, however, was reported. We have been unable to convert MCR_{ox2} to the MCR_{red1} state. Further comparisons between MCR_{ox1} and MCR_{ox2} may provide insights into the structural environment of Ni F_{430} in MCR.

In summary, the discovery of an alternative method for preparing the MCR_{ox1} ready state that can be converted to the MCR_{red1} active state will help make investigations into the catalytic mechanism of methane formation from methyl-SCoM and HS-CoB feasible. The ready state appears to contain a Ni−S bond although further experiments are required to confirm if a mercapto group is ligated to the Ni in MCR_{ox1}. Activation of MCR may labilize this proposed Ni−S bond.

ACKNOWLEDGMENT

We thank Prof. Rolf Thauer and Dr. Marcel Goubeaud for their helpful comments and for sharing a copy of their manuscript (*21*) four months prior to publication during the development of this protocol for generating MCR_{ox1}.

NOTE ADDED IN PROOF

While this paper was under review, a high resolution crystal structure of the *Mb. thermoautotrophicum* MCR was published [Ermler, U., Grabarse, W., Shima, S., Goubeaud, M., and Thauer, R. K. (1997) *Science 278*, 1457−1462].

REFERENCES

1. DiMarco, A. A., Bobik, T. A., and Wolfe, R. S. (1990) *Annu. Rev. Biochem. 59*, 355.
2. Ferry, J. G. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 473−502.
3. Ferry, J. G. (1993) in *Chapman & Hall Microbiology Series* (Reddy, C. A., Chakrabarty, A. M., Demain, A. L., and Tiedje, J. M., Eds.) pp 536, Chapman & Hall, London.
4. Deppenmeier, U., Muller, V., and Gottschalk, G. (1996) *Arch. Microbiol. 165*, 149−163.
5. Thauer, R. (1997) *Antonie van Leeuwenhoek 71*, 21−32.
6. Rouviére, P. E., and Wolfe, R. S. (1988) *J. Biol. Chem. 263*, 7913−7916.
7. Gorris, L. G. M., and van der Drift, C. (1994) *Biofactors 4*, 139−145.
8. Ellermann, J., Hedderich, R., Böcher, R., and Thauer, R. K. (1988) *Eur. J. Biochem. 172*, 669−677.
9. Hartzell, P. L., and Wolfe, R. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6726−6730.
10. Farber, G., Keller, W., Kratky, C., Jaun, B., Pfaltz, A., Spinner, C., Kobelt, A., and Eschenmoser, A. (1991) *Helv. Chim. Acta 74*, 697−716.
11. Jablonski, P. E., and Ferry, J. G. (1991) *J. Bacteriol. 173*, 2481−2487.
12. Albracht, S. P. J., Ankel-Fuchs, D., Böcher, R., Ellermann, J., Moll, J., van der Zwann, J. W., and Thauer, R. K. (1988) *Biochim. Biophys. Acta 941*, 86.
13. Rospert, S., Bocher, R., Albracht, S. P. J., and Thauer, R. K. (1991) *FEBS Lett. 291*, 371−375.
14. Berkessel, A. (1991) *Bioorg. Chem. 19*, 101−115.
15. Cha, M., Shoner, S. C., and Kovacs, J. A. (1993) *Inorg. Chem. 32*, 1860−1863.
16. Ahn, Y., Krzycki, J. A., and Floss, H. G. (1991) *J. Am. Chem. Soc. 113*, 4700−4701.
17. Zilbermann, I., Golub, G., Cohen, H., and Meyerstein, D. (1994) *Inorg. Chim. Acta 227*, 1−3.
18. Helvenston, M., and Castro, C. (1992) *J. Am. Chem. Soc. 114*, 8490−8496.
19. Holliger, C., Pierik, A. J., Reijerse, E. J., and Hagen, W. R. (1993) *J. Am. Chem. Soc. 115*, 5651−5656.
20. Hartzell, P. L., Escelante-Semerena, J. C., Bobik, T. A., and Wolfe, R. S. (1988) *J. Bacteriol. 170*, 2711.
21. Goubeaud, M., Schreiner, G., and Thauer, R. K. (1997) *Eur. J. Biochem. 243*, 110−114.
22. Gunsalus, R. P., Romesser, J. A., and Wolfe, R. S. (1978) *Biochemistry 17*, 2374−2377.

23. Noll, K. M., Rinehart, K. L., Jr., Tanner, R. S., and Wolfe, R. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4238−4242.
24. Bobik, T. A., and Wolfe, R. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 60−63.
25. Sowers, K. R., Nelson, M. F., and Ferry, J. G. (1984) *Curr. Microbiol. 11*, 227−230.
26. Wolin, E. A., Wolin, M. J., and Wolfe, R. S. (1963) *J. Biol. Chem. 238*, 2882−2886.
27. Jablonski, P. E., DiMarco, A. A., Bobik, T. A., Cabell, M. C., and Ferry, J. G. (1990) *J. Bacteriol. 172*, 1271−1275.
28. Zinder, S. H., and Mah, R. A. (1979) *Appl. Environ. Microbiol. 38*, 996−1008.
29. Schonheit, P., Moll, J., and Thauer, R. K. (1980) *Arch. Microbiol. 127*, 59−65.
30. Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
31. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
32. Margoliash, E., and Schejter, A. (1966) *Adv. Protein Chem. 21*, 113.
33. Pfaltz, A., Juan, B., Fassler, A., Eschenmoser, A., Jaenchen, R., Gilles, H. H., Diekart, G., and Thauer, R. K. (1982) *Helv. Chim. Acta 65*, 828−865.
34. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, San Francisco.
35. Henderson, P. J. F. (1972) *Biochem. J. 127*, 321.
36. Daniels, L., Belay, N., and Rajagopal, B. S. (1986) *Appl. Environ. Microbiol. 51*, 703−706.
37. Ellermann, J., Rospert, S., Thauer, R. K., Bokranz, M., Klein, A., Voges, M., and Berkessel, A. (1989) *Eur. J. Biochem. 184*, 63−68.
38. Brenner, M. C., Zhang, H., and Scott, R. A. (1993) *J. Biol. Chem. 268*, 18491−18495.
39. Jaun, B., and Pfaltz, A. (1986) *J. Chem. Soc., Chem. Commun. 17*, 1327−1329.
40. Telser, J., Fann, Y. C., Renner, M. W., Fajer, J., Wang, S. K., Zhang, H., Scott, R. A., and Hoffman, B. M. (1997) *J. Am. Chem. Soc. 119*, 733−743.
41. Shiemke, A. K., Kaplan, W. A., Hamilton, C. L., Shelnutt, J. A., and Scott, R. A. (1989) *J. Biol. Chem. 264*, 7276−7284.
42. Procyk, A. D., Stolzenberg, A. M., and Bocian, D. F. (1993) *Inorg. Chem. 32*, 627−633.
43. Balch, W. E. (1979) Ph.D. Thesis, University of Illinois, Urbana−Champaign.
44. Fernandez, V. M., Hatchikian, E. C., Patil, D. S., and Cammack, R. (1986) *Biochem. Biophys. Acta 883*, 145−154.
45. Shiemke, A. K., Shelnutt, J. A., and Scott, R. A. (1989) *J. Biol. Chem. 264*, 11236−11245.
46. Kaplan, W. A., Suslick, K. S., and Scott, R. A. (1991) *J. Am. Chem. Soc. 113*, 9824−9827.
47. Lin, S.-K., and Jaun, B. (1991) *Helv. Chim. Acta 74*, 1725−1738.

BI972145X