Spectroscopic and Kinetic Studies of the Reaction of Bromopropanesulfonate with Methyl-coenzyme M Reductase

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Methyl-coenzyme M reductase (MCR) catalyzes the final step of methanogenesis in which coenzyme B and methyl-coenzyme M are converted to methane and the heterodisulfide, CoMS-SCoB. MCR also appears to initiate anaerobic methane oxidation (reverse methanogenesis). At the active site of MCR is coenzyme F₄₃₀, a nickel tetrapyrrole. This paper describes the reaction of methyl-coenzyme M (methyl-SCoM) with N₇-mercaptoheptanoyl-threonine phosphate (HSCoB) to generate methane and the mixed disulfide CoBS-SCoM (Reaction 1) (1, 2). HSCoB serves as the two-electron donor (3). At the active site of MCR is a nickel tetrapyrrolic cofactor called coenzyme F₄₃₀ that is central to catalysis (4–6). X-ray crystallographic studies reveal that coenzyme F₄₃₀ binds noncovalently to MCR at the bottom of a 30-Å hydrophobic channel (7). The phosphate group of HSCoB binds at the upper lip of this channel with its thiol group located 8.7 Å from the central nickel atom of F₄₃₀.

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\text{Methyl-SCoM} + \text{HSCoB} \rightarrow \text{CH}_4 + \text{CoBS-SCoM}
\]

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\Delta G = -30 \text{ kJ/mol}
\] (Eq. 1)

MCR can exist in several states that differ in their nickel oxidation and/or coordination states (Fig. 1). The MCRred₁ state is catalytically active (8–10) based on the correlation between the amount of this enzyme form and MCR activity in Methanothermobacter marburgensis (10) and Methanosarcina thermophila (11). MCRox₁ appears to be the only state that can be converted in vitro to active MCRred₁, which is accomplished by incubating the MCRox₁ state with a strong reductant, titanium(III) citrate (Ti(III) citrate) (10). The MCRred₁ state also can be generated in vivo when cells are bubbled with 100% H₂ before harvesting (8). MCRox₁ can be formed when the gas medium of growing cells is switched before harvesting from 80% H₂, 20% CO₂ to 80% N₂, 20% CO₂ (8) or by treating the growing cells with sodium sulfide (11).

High resolution crystal structures for three EPR-silent and inactive Ni(II) states of this enzyme have been determined: MCRsilent, MCRox₁-silent, and MCRred₁-silent (7, 12, 13). These states have several common features: the central nickel atom of MCR is catalytically active and binds noncovalently to MCR at the bottom of a 30-Å hydrophobic channel (7).

\[\text{REACTION 1}\]

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\text{Methyl-SCoM} + \text{HSCoB} \rightarrow \text{CH}_4 + \text{CoBS-SCoM}
\]

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\Delta G = -30 \text{ kJ/mol}
\] (Eq. 1)
F430 is coordinated by four planar tetapyrrole nitrogen atoms and a lower axial oxygen ligand contributed by the carbonyl oxygen of the side chain of Gln-147. In the Ni(II)silent form of MCR, the upper axial nickel ligand is the sulfonate oxygen of CoBS-SCoM, whereas, in the Ni(II)ox1-silent form, this site is occupied by the thiol(ate) group of CoM-S(2H) (Fig. 1) (7, 12, 13). A five-coordinate form of Ni(II)-MCRred1-silent, lacking an upper axial ligand, has also been observed in the crystal structure (13). The structures of “ready” MCRox1 and “active” MCRred1 forms have not yet been determined, since they are quite labile. Based on an array of spectroscopic (x-ray absorption (14), UV-visible (15, 16), EPR (14, 17), pulsed EPR (17), and magnetic circular dichroism (15, 16)) and computational methods (15), MCRox1 is best described as a high spin Ni(II) coupled to a thiyl-radical (15, 17) (Fig. 1). MCRred1 is known to be in the Ni(I) state (9–11). Recent x-ray absorption results (14, 18) indicate that the nickel is six-coordinate in the MCRox1 state, yet there is still some controversy about the MCRred1 state, which is either five-coordinate with an open upper axial site (14) or six-coordinate with two axial ligands coordinated through an oxygen atom (18) (Fig. 1). The coordination state of MCRred1 needs to be resolved, because having an open upper axial site would poise the Ni(I) for reaction with substrate, whereas an axial ligand would need to be replaced/removed before reaction could occur. Kinetic studies using several small molecules (CHCl3, CH2Cl2, and NO) and substrate analogs (including 3-bromopropanesulfonate (BPS)) support the requirement for ligand exchange (19); however, further studies are important in resolving this mechanistic question.

Two radically different mechanisms have been proposed for the catalytic mechanism of MCR. In Mechanism I, Ni(I)-MCRred1 reacts with the methyl group of methyl-SCoM, forming a methyl-Ni(III) or methyl-Ni(II) intermediate (20–23). Although the corresponding methyl-nickel species has not been observed on the enzyme, methane formation from the reaction of Ni(I)-derivatives of F430 and activated methyl donors like methylsulfonium ions has been observed (24), and a methyl-Ni(II) form of the pentamethyl ester of F430 has been characterized by NMR methods (25). Furthermore, reacting Ni(I)-octaethylisobacteriochlorin (a structural cousin of F430) with various alkyl halides generates alkyl-Ni(III) species that undergo reduction to the alkyl-Ni(II) followed by protonolysis to yield the corresponding alkane (26).

On the basis of a computational study using density function theory (DFT), the proposed methyl-nickel species was considered to not be a feasible intermediate in methane synthesis, and Mechanism II was proposed as an alternative to Mechanism I (27). The key steps of Mechanism II include nucleophilic attack by Ni(I)-MCRred1 on methyl-SCoM to form a nickel-thiolate complex and a methyl radical, which abstracts a hydrogen atom from HSCoB to generate methane (27).

Unfortunately, it has been difficult to distinguish between these mechanisms, mainly because we have so far been unable to observe any spectrally distinct intermediates by rapid kinetics. Another complication is that MCRred2-catalyzed cleavage of the C–S bond of methyl-SCoM requires the other substrate, HSCoB, even under single turnover conditions (23). In the studies described here, we used BPS as a substrate analog, because, unlike methyl-SCoM, reduction of BPS in a single turnover reaction does not require the other substrate HSCoB; furthermore, EPR and UV-visible changes can be observed. When MCRred1 and MCRred2 are incubated with BPS, their EPR signals convert to the MCRPS signal, which does not exhibit measurable halogen-Ni(I) interaction (28) and has been assigned to a high spin Ni(II)/alkyl radical species (29). Here we show that this EPR spectroscopic change is accompanied by formation of an “MCRox1-like” UV-visible spectrum and the reduction of BPS to propanesulfonate.

**EXPERIMENTAL PROCEDURES**

*Materials and Organisms—M. marburgensis* (f. *M. thermoautotrophicum* strain Marburg) was obtained from the Oregon

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4 In these reactions, a stoichiometry of 2 Ni(I)/alkyl halide was observed, indicating reduction of the alkyl-Ni(III) to the alkyl-Ni(II) state before protonolysis.

5 Y.-C. Horng and S. W. Ragsdale, unpublished observations.
Collection of Methanogens catalogue as OCM82. All buffers, media ingredients, and other reagents were acquired from Sigma and, unless otherwise stated, were of the highest purity available. Solutions were prepared using Nanopure deionized water. N₂ (99.98%), H₂S (99.0%), CO (99.99%), argon (99.8%), H₂/CO₂ (80%/20%), and ultrahigh purity H₂ (99.999%) were obtained from Linveld (Lincoln, NE).

Methyl-SCoM was prepared from coenzyme M and methyl iodide (30). After methyl-SCoM was crystallized twice in acetone, its purity was checked by ¹H NMR spectroscopy. CoBS-SCoB was prepared as described from 7-bromoheptanoic acid (Karl Industries, Aurora, OH), N-hydroxysuccinimide, and α-phospho-ε-threonine (31, 32). HSCoB was generated from CoBS-SCoB by anaerobic reduction with NaBH₄ (33), and its purity was ascertained by HPLC. The concentration of HSCoB was checked routinely with Ellman’s reagent (34) before using it. 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 weight % solution in 2 N hydrochloric acid) (Acros Organics, Morris Plains, NJ) under anaerobic conditions and adjusting the pH to 7.0 with sodium bicarbonate (35). The concentration of Ti(III) citrate was determined routinely by titrating a 50-ml ultrafiltration stirred cell (Amicon; Millipore Corp., Bedford, MA) with a 30 kDa molecular mass cut-off filter using high pressure argon that had been passed through an oxisorb column (Oxyclear; Supelco) to remove oxygen.

MCR_red1 used for the experiments presented in Figs. 2, 5, 8, 9, S1, and S2 was purified as described with the following modifications (10). For experiments requiring high concentrations of MCR, MCR_ox1 was concentrated in a 50-ml ultrafiltration stirred cell (Amicon; Millipore Corp., Bedford, MA) with a 30 kDa molecular mass cut-off filter using high pressure argon that had been passed through an oxisorb column (Oxyclear; Supelco) to remove oxygen.

MCR_ox1 was purified as described, except that methyl-SCoM was omitted from the lysis buffer (10). For experiments requiring high concentrations of MCR, MCR_ox1 was concentrated in a 50-ml ultrafiltration stirred cell (Amicon; Millipore Corp., Bedford, MA) with a 30 kDa molecular mass cut-off filter using high pressure argon that had been passed through an oxisorb column (Oxyclear; Supelco) to remove oxygen.

Spectroscopy of MCR—UV-visible spectra of MCR were recorded in the anaerobic chamber on ice packs in Styrofoam containers. In this way, MCR_red1 can be stored in the anaerobic chamber for 3–4 months before losing all activity. This purification method routinely generates 50–80% MCR_red1 as determined by UV-visible and EPR spectroscopy.

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). EPR spectra were recorded on a Bruker ESP 300E spectrometer recently upgraded to an EMX, equipped with an Oxford ITCC4 temperature controller, a Hewlett-Packard model 5340 automatic frequency counter, and Bruker gaussmeter. Unless otherwise noted, the EPR spectroscopic parameters included the following: temperature, 100 K; microwave power, 10 milliwatts; microwave frequency, 9.43
MCR Reduction of Bromopropanesulfonate

GHG; receiver gain, $2 \times 10^4$; modulation amplitude, 12.8 G; modulation frequency, 100 kHz. Double integrations of the EPR spectra were performed and referenced to a 1 mm copper perchlorate standard. All NMR data were acquired at 298 K on a Bruker Avance DRX 500-MHz NMR instrument (Bruker Biospin Corp., Billerica, MA) equipped with a TXI cryoprobe in the UNL Chemistry Department.

Conversion of MCR$_{ox1}$ to MCR$_{red1}$—For the experiments presented in Figs. 3, 4, 6, and 7, MCR$_{ox1}$ was activated to the MCR$_{red1}$ state in the absence of methyl-SCoM or HSCoM in a reaction mixture containing 20 mM Ti(III) citrate, 0.67 M TAPS (pH 10), 16.7 mM Tris-Cl (pH 7.6), and MCR (30 – 200 μM). The mixture was heated at 60°C for 40 min, cooled on ice, and then neutralized to pH 7.0 – 7.1 by adding an equal volume of 2 M Tris-HCl buffer (pH 7.0) (11). Protein concentrations were determined by the Bradford method using the Bio-Rad reagent and bovine serum albumin as a standard (38). F$_{330}$ content was estimated using an extinction coefficient of 22,000 cm$^{-1}$ M$^{-1}$ at 420 nm (39).

Conversion of MCR$_{red1}$ to MCR$_{ps}$—MCR$_{red1}$ used in Figs. 8, 9, and S1 was incubated for 5 min with a 10-fold excess of BPS in 50 mM Tris, pH 7.6. Unreacted BPS was removed from MCR$_{ps}$ by buffer exchange using Amicon Ultra-15 centrifuge filter units with a 50 kDa cut-off (Millipore). Typically, 300 – 600 μl of MCR$_{ps}$/BPS reaction mixture was exchanged into 3 – 6 ml of 50 mM Tris, pH 7.6. This mixture was concentrated to 200 – 300 μl, and this process was repeated three times. The EPR spectroscopic parameters were as noted above except the following: temperature, 70 K; microwave power, 10.2 milliwatts; modulation amplitude, 5.0 G; scans, 4.

Measurement of MCR Activity—MCR assays were performed at 65°C in rubber-sealed 8-ml screw vials. The standard assay mixture contained 20 mM methyl-SCoM, 1.2 mM HSCoB, 0.6 mM aquacobalam, 25 mM Ti(III) citrate, and 0.5 M MOPS (pH 7.0) in a final volume of 0.4 ml. The reaction was started by increasing the temperature from 4 to 65°C. The methane generated was determined by withdrawing gas samples at specific time points for analysis by gas chromatography (Varian model 3700 equipped with a flame ionization detector). Alternatively, MCR activity was measured by following the time-dependent loss of radioactivity as $^{14}$CH$_3$-SCoM was converted to $^{14}$C-methane (23). 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 min$^{-1}$.

Reactions between MCR$_{red1}$ and Sulfonyl Group—MCR$_{red1}$ (typically 0.1 – 0.2 mm) was incubated in 0.5 M TAPS (pH 10.0) for 10 min with various sulfonyl-containing compounds at concentrations between 1 mm and 2 μM: 2-bromoethanesulfonic acid sodium salt (BES), 2-(N-morpholinio)ethanesulfonate (MES), BPS, 3-chloropropanesulfonyl chloride, CAPS, 3-mercapto-1-propanesulfonic acid sodium salt (Fluka), 4-bromo-1butanesulfonic acid sodium salt (BBS), 4-chlorobutyric acid, 1-propanesulfonic acid sodium salt monohydrate (Fluka), 1-butanesulfonic acid sodium salt (Fluka), and MOPS. The formation of new complexes was identified by EPR.

Stopped-flow Studies—Stopped-flow experiments were performed on an Applied Photophysics spectrophotometer (SX.MV18; Leatherhead, UK) equipped with a photodiode array detector. Constant temperature was maintained with a bath of nitrogen-bubbled water from a circulating pump to maintain anaerobicity. Rigorous measures were taken to purge oxygen from the stopped-flow instrument. The solutions of enzymes and inhibitors were made in the anaerobic chamber in 0.5 M TAPS, pH 10.0. The solutions were then loaded into tonometers, which had been incubated in the anaerobic chamber for 4 days and served as reservoirs for the drive syringes of the stopped-flow instrument. The drive syringes were maintained anaerobically at 25°C in a temperature-controlled bath of anaerobic water. MCR$_{red1}$ and varied concentrations of BPS were rapidly mixed at 25°C in a 1:1 ratio. The reaction was monitored in the single wavelength mode by following the decay of MCR$_{red1}$ at 388 and 715 nm, and MCR$_{ps}$ formation was followed at 422 nm. Data were fit to single exponential decay functions with software provided by Applied Photophysics (version SX.MV.18). Reported rate constants are the average of at least five different rapid mixing experiments.

HPLC Connected to Conductivity Detector—The reaction between MCR$_{red1}$ and BPS was initially followed by HPLC using a 3.9 x 150-mm μBondapak C$_{18}$ analytical column (Waters, Milford, MA), which was developed with a 0 – 80% methanol gradient in 10 mM ammonium formate (pH 3.3) (30 min at a flow rate of 1 ml/min). BPS decay was monitored using an absorbance detector set at 207 nm, whereas product formation was followed using a conductivity detector (Wescan Instruments Inc., Deerfield, IL). In this experiment, after activation in 0.5 M TAPS (pH 10.0), the MCR solution was exchanged with a solution containing 50 mM Tris-HCl (pH 7.6), 2 mM Ti(III) citrate using a 50-ml ultrafiltration stirred cell (Amicon) with a 30 kDa molecular mass cut-off. This step also removed any free F$_{330}$ that could have been released from the enzyme during the activation step. The reaction was started by injecting 20 μl of 0.5 mM BPS solution (50 mM Tris-HCl, pH 7.6) into the 2-ml enzyme mixture. At each time point, 200 μl of reaction mixture was removed, frozen in EPR tubes, and observed by EPR spectroscopy. Next, the enzyme solution was transferred to a 1.5-ml microcentrifuge tube and exposed to O$_2$ for 30 min. After diluting the sample with the same amount of Tris-HCl buffer, enzyme and ligands were separated using microconcentrators (Amicon) with 30 kDa molecular mass cut-off. The clear filtrate, which lacked MCR, was injected directly into the HPLC with the conductivity detector. The C$_{18}$ column was pre-equilibrated with 50 mM formic acid (pH 3.0) and eluted with the same buffer at a flow rate of 0.5 ml/min. The retention times for BPS and PS were 6 and 5 min, respectively.

Chemical Quench Studies—Chemical quench experiments presented in Fig. 6 were performed at 20°C using an Update Instruments (Madison, WI) chemical/freeze-quench apparatus with a model 745 controller. Rapid reaction kinetic studies were performed with MCR$_{red1}$ and BPS in separate 2-ml stopped-flow syringes. Solutions of MCR$_{red1}$ and BPS (both in buffer containing 50 mM Tris-HCl, pH 7.6, and 5 mM Ti(III) citrate) were rapidly mixed by activating the ram to displace each syringe by 1.3 mm, generating a total reaction volume of 82 μl (41 μl from each syringe) per shot. The ram speed was varied from 0.8 to 8.0 cm/s and was shown not to affect the observed reaction rate. Typically, a ram speed of 4.0 cm/s was used with...
a 100-ms aging hose. For each data point, six shots were collected (492 μl) in an 18-ml scintillation vial containing 0.2 ml of 0.5 N formic acid. After the chemical quench experiment, each sample was lyophilized and dissolved in 100 μl of deionized water. The time course of each reaction was followed by monitoring the decrease in concentration of BPS and the formation of PS. The conditions and profile for eluting PS and BPS are the same as described above except that 80% methanol was used to wash out F₄₃₀ from the column after each run. Data were fit to single exponential equations using Sigma Plot (Point Richmond, CA).

MCRred₁ activity was measured prior to the rapid reaction kinetic experiments. The typical specific activity of the MCR used for the stopped-flow or chemical quench studies was 20–25 units/mg at 65 °C, which would be equivalent to 60–100 units/mg when corrected to 100% active MCRred₁. The amount of MCRred₁ in each rapid reaction kinetic experiment was measured by double integration of the MCRred₁ EPR signal before and after the stopped-flow or chemical quench experiments. Typical spin concentrations of the MCRred₁ in the MCR samples for stopped-flow or chemical quench experiment ranged from 0.25 to 0.35 spin/mol of MCR, and less than 10% loss of this state occurred during the experiment.

MCRred₁ used in the chemical quench studies presented in Fig. 5 was prepared by removing HSCoM by buffer exchange exchanged into 50 mM Tris, pH 7.6, 0.1 m M Tr(III) citrate with Amicon Ultra-15 centrifuge filter units with a 50 kDa cut-off (Millipore). The concentration of MCRred₁ was determined by UV-visible spectroscopy using extinction coefficients of 27.0 mM⁻¹ cm⁻¹ and 9.15 mM⁻¹ cm⁻¹ at 385 and 420 nm, respectively, using a multiple wavelength calculation (40). These values were established from a 100% MCRred₁ sample as determined by EPR using a 1 M copper perchloric acid standard. The concentration of MCRsilent was calculated using extinction coefficients of 22.0 and 12.7 mM⁻¹ cm⁻¹ at 420 and 385 nm, respectively (39). To ensure the accuracy of this method for determining the amount of MCRred₁ in a solution containing a mixture of MCRred₁ and MCRsilent, forms of the enzyme, the total protein concentration calculated with this method was compared with values determined using other methods. Protein concentrations calculated by this method were in good agreement (10–15%) with the Bio-Rad reagent (Bio-Rad) using bovine serum albumin as a standard. Total protein concentrations were also measured by converting a heterogeneous mixture containing MCRred₁ + MCRsilent to a homogeneous mixture containing 100% MCRsilent by exposing it to air and using an extinction coefficient of 22.0 mM⁻¹ cm⁻¹ to calculate the total MCR concentration (39).

Chemical quench experiments for Fig. 5 were performed with MCRred₁ and BPS in separate 2-ml stopped-flow syringes. Solutions of MCRred₁ and BPS were rapidly mixed by activating the ram to displace each syringe by 6.5 mm, generating a total reaction volume of 413 μl (206.5 μl from each syringe) per shot. A ram speed of 1.25 cm s⁻¹ was used with a 500-ms aging hose. Each spectrum represents a total of 10 shots collected in a 30-ml serum vial (Alltech) containing 3 ml of 0.5 N formic acid to quench the reaction. After the reaction was quenched, enzyme and ligands were separated using a 50-ml ultrafiltration stirred cell concentrator fitted with a 50 kDa cut-off filter (Millipore). To separate BPS/PS from other ligands, mainly F₄₃₀, the flow-through from the stirred cell was applied to a 2-ml C₁₈ column equilibrated with water. The flow-through from the C₁₈ column was collected and lyophilized to dryness and dissolved in 550 μl of 100% D₂O/0.75% 3-(trimethylsilyl)-propionic acid-D₄ by weight for NMR analysis.

RESULTS

Reaction of MCRred₁ with BPS and Related Analogs, Studied by EPR—BPS is the most potent known inhibitor of MCR (41). When MCRred₁ is reacted with BPS, a unique EPR signal called “MCRΔBPS” is observed, which, because of its air sensitivity and its similarity to the MCRred₁ spectrum, was assigned as an Ni(I) state (42). A more recent study based on EPR data suggests that when MCRred₁ reacts with BPS, bromide (or HB₈) is presumably released, and a species is formed that can be described as an Ni(III)-propylsulfonate or a high spin Ni(II) with an alkylsulfonyl radical (29). We confirmed this result (Table 1 and Fig. 2, inset) and have studied this reaction by various kinetic and spectroscopic methods (Reaction 2). Because the bromide has presumably undergone elimination, the designation “MCRΔBPS” is misleading, and we use the designation “MCRPS” instead. When MCRred₁ is incubated with other structurally related sulfonylates, an EPR signal nearly identical to MCRPS is observed (Table 1) (29). Compounds that elicit this spectral change include 3-chloropropanesulfonyl chloride (1 mm) and BBS (7 mm) (Table 1). Unlike MCRox₁ and MCRred₁, these “MCRΔPS-like” EPR signals show no resolved hyperfine splitting, even when the modulation amplitude is decreased to 5.0 G. The MCRPS signal is relatively stable in the absence of oxygen or one of the reagents that react with it (below), decaying with a half-life of ~8 h at pH 7.6 in the anaerobic chamber. However, in the presence of oxygen, the signal is rapidly quenched within 5 min).

\[ \text{MCRred₁} + \text{BPS} \rightarrow \text{MCRPS} + \text{Br⁻} \]

**REACTION 2**

The requirement for the sulfonate group of BPS to elicit the MCRPS EPR signal was tested using 4-chlorobutyrate, in which a carboxylate group replaces the sulfonate. When reacted with MCRred₁, this BPS-like analog gave rise to an EPR signal nearly identical to MCRPS. A similar result was reported earlier using 4-bromobutyric acid (29). However, reaction of MCRred₁ with propanesulfonate (70 mM), butanesulfonate (70 mM), and 3-mercaptop-1-propanesulfonate (90 mM) neither decreased the MCRred₁ EPR signal, as reported previously (29) (Table 1).
pounds, BPS as a starting material for MOPS and CAPS and BES for MES and CHES (43). This would explain the high concentrations needed to elicit the MCR<sub>PS</sub> signal, and the discrepancy in results from different batches of the same compound. For example, incubation of MCR<sub>red1</sub> with 0.5 M of 98% pure CAPS at pH 7.0 elicited the MCR<sub>PS</sub> EPR signal, whereas incubating with 0.5 M 99% pure CAPS at pH 7.0 did not. Therefore, we suggest that the EPR signals observed upon incubating MCR<sub>red1</sub> with MOPS or CAPS and quenching of the EPR signal upon incubating with MES or CHES is due to contamination from the BPS or BES remaining in the starting material, respectively.

**Reaction of MCR<sub>red1</sub> and MCR<sub>PS</sub> Formation, Followed by Rapid Kinetics**—The UV-visible absorption spectra of EPR-active MCR<sub>PS</sub> (Fig. 2) resemble those of EPR-active MCR<sub>ox1</sub> and EPR-silent Ni(II) MCR<sub>silent</sub> with absorbance maxima at 423 nm. In contrast, MCR<sub>red1</sub> and Ni(I)F<sub>430</sub> exhibit a 40-nm blue shift relative to the MCR<sub>PS</sub>, MCR<sub>ox1</sub>, and Ni(II) forms, which suggest that BPS causes a redox change in MCR. We followed the reaction of MCR<sub>red1</sub> with BPS by absorption spectroscopy using a stopped-flow instrument (Fig. 3). Single exponential kinetics are observed although the reaction is not run under pseudo-first order conditions. Decay of the MCR<sub>red1</sub> absorbance peaks at 388 and 715 nm matches the rate of formation of the MCR<sub>PS</sub> absorbance peak at 422 nm. This indicates that there is no intermediate between the MCR<sub>red1</sub> and MCR<sub>PS</sub> states; alternatively, any intermediate that is formed is too transient to be observed. On the basis of the BPS concentration dependence of the UV-visible spectral changes (Fig. 4), the second order rate constant for MCR<sub>PS</sub> formation (or MCR<sub>red1</sub> decay) is 1.6 × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup> (at 20 °C). This value is about 10-fold higher than the k<sub>cat</sub>/K<sub>m</sub> for methyl-SCoM (1.9 × 10<sup>4</sup> M<sup>−1</sup> s<sup>−1</sup> at 65 °C) and 100-fold larger than the k<sub>cat</sub>/K<sub>m</sub> for HSCoB (2.2 × 10<sup>3</sup> M<sup>−1</sup> s<sup>−1</sup>; 20 °C) in methane formation (23). The rate constants at pH 7.6 (in 50 mM Tris-HCl buffer) are identical to those at pH 10.0 (in 0.5 M TAPS) (data not shown).

MCR<sub>red1</sub> can also react with BBS but with significantly lowered efficiency. As shown in Fig. S2 (see supplemental material), the kinetic parameters for formation of MCR<sub>PS</sub> (in analogy with MCR<sub>PS</sub>) and MCR<sub>red1</sub> decay are as follows: k<sub>max</sub> = 21.4 ± 0.3 s<sup>−1</sup>, K<sub>m</sub> = 9.4 ± 0.3 mM, k<sub>max</sub>/K<sub>m</sub> = 2270 ± 70 M<sup>−1</sup> s<sup>−1</sup>. Thus, the second order rate constant for reaction between MCR<sub>red1</sub> and BBS is about 70-fold lower than that for reaction with BPS. In the case of BBS, the electrophilic carbon adjacent to the bromide leaving group is five bonds (∼6 Å) from the sulfonate group, which corresponds to the methyl group of methyl-SCoM.

**Reaction of MCR<sub>ox1</sub> with BPS**—When MCR<sub>ox1</sub> was incubated with BPS in the absence of Ti(III) citrate for several hours at room temperature, the MCR<sub>PS</sub> EPR signal appeared. The spectrum of MCR<sub>PS</sub> formed from MCR<sub>ox1</sub> is indistinguishable from that formed from MCR<sub>red1</sub>, yet the second order rate constant
(2.4 s\(^{-1}\)) (19) is 10\(^5\)-fold slower than that for formation of MCR\(_{\text{PS}}\) from MCR\(_{\text{red1}}\) (see above). This suggests, as proposed earlier (44), that the reactivity of MCR\(_{\text{ox1}}\) may be due to a very low amount (as low as 0.001%; i.e. 1/10\(^5\)) of MCR\(_{\text{red1}}\) present in the MCR\(_{\text{ox1}}\) solution. Such low amounts would be undetectable by any spectroscopic method. The rate of MCR\(_{\text{ox1}}\) decay matches the rate of MCR\(_{\text{PS}}\) formation, and these rates are faster at pH 10.0 than at pH 7.6 (data not shown) (40), which also would be consistent with MCR\(_{\text{red1}}\) intermediacy in this process, since pH 10.0 is optimal for activation of MCR\(_{\text{ox1}}\) to MCR\(_{\text{red1}}\) with Ti(III) citrate (45). Furthermore, as described above, formation of MCR\(_{\text{PS}}\) involves oxidation of MCR\(_{\text{red1}}\) to an “MCR\(_{\text{ox1}}\)-like” state and reduction of BPS (see above). Thus, the data indicate that MCR\(_{\text{red1}}\) but not MCR\(_{\text{ox1}}\) can generate the MCR\(_{\text{PS}}\) state.

MCR\(_{\text{red1}}\)-catalyzed Propanesulfonate Formation from BPS—As noted above, the similarity between the UV-visible and EPR spectra of MCR\(_{\text{PS}}\) and MCR\(_{\text{ox1}}\) indicates that MCR\(_{\text{red1}}\) is two electrons more reduced than MCR\(_{\text{PS}}\). The EPR spectra generated when MCR\(_{\text{red1}}\) is reacted with 3-iodo- or 3-chloropropanesulfonate is identical to MCR\(_{\text{PS}}\) with no detectable hyperfine splitting from bromide or iodine (28), indicating that the halogen has been eliminated during formation of the MCR\(_{\text{PS}}\) species. Rapid chemical quench methods were used to identify the alkylsulfonate group that is proposed to ligate to nickel (see Scheme 1). It was expected that quenching the reaction in acid would protonate the propylsulfonate ligand (forming HPS) and inactivate MCR. We removed HSCoM from the solution containing MCR\(_{\text{red1}}\) (MCR\(_{\text{red1}}\) is usually stored in the presence of HSCoM, because it stabilizes this form of the enzyme), because thiolytes react directly with BPS in the absence of MCR. MCR\(_{\text{red1}}\) was rapidly mixed with BPS in the absence of HSCoB and HSCoM under single turnover conditions in the chemical quench apparatus, and the reaction was quenched into a solution containing 0.5 M formic acid. Then the small molecule products were separated from the protein by ultrafiltration and analyzed by NMR spectroscopy to measure the appearance of the product of this reaction. When MCR\(_{\text{red1}}\) and Ti(III) citrate (in a 2.4- and 1.4-fold molar excess of the BPS, respectively) were reacted with BPS, HPS was detected as the product of the acid quench (Reaction 3; Fig. 5A). The reaction has a strict requirement for MCR\(_{\text{red1}}\), since, when MCR\(_{\text{red1}}\) was omitted and Ti(III) citrate was present in a 2.9-fold molar excess (relative to BPS), the HPS peaks were absent, whereas peaks characteristic of BPS remained (Fig. 5B). Furthermore, when the MCR\(_{\text{silent}}\) form of
MCR Reduction of Bromopropanesulfonate

To determine the rate of propylsulfonate adduct formation relative to the rate of MCR\textsubscript{PS} generation (as measured by UV-visible and EPR spectroscopy), the MCR\textsubscript{red1} catalyzed conversion of BPS to PS was monitored under single turnover conditions by rapid chemical quench methods (quenched with formic acid). HPS formation and BPS depletion were measured by HPLC connected to a conductivity detector (Fig. 6). The product was identified by its elution at the same position as an authentic HPS standard. The rates and amplitudes for BPS decay and HPS formation were identical and equaled the rates of MCR\textsubscript{PS} formation and MCR\textsubscript{red1} decay (above in Fig. 3).

These combined results indicate that the reaction of MCR\textsubscript{red1} with BPS occurs according to Reaction 3 (i.e. the oxidation of MCR\textsubscript{red1} is coupled to reduction of BPS to form MCR\textsubscript{PS}, which undergoes protonolysis to form propanesulfonate). Propanesulfonate appears to be the only product formed from BPS, as summarized in Scheme 1.

Evidence that MCR\textsubscript{PS} Can Be Reactivated to MCR\textsubscript{red1}—The results described above demonstrate that Ni(I) of MCR\textsubscript{red1} performs a nucelophilic attack on BPS to form MCR\textsubscript{PS} and bromide. This is an oxidative addition, much like the methyltransferase-catalyzed reaction of cob(I)alamin with CH\textsubscript{3}-H\textsubscript{4}-folate to form methyl-cob(III)alamin and H\textsubscript{4}-folate (46). The reaction of MCR with BPS has been thought to lead to irreversible inactivation of MCR. However, NMR results indicate that multiple turnovers of BPS conversion to propanesulfonate can occur.

PS formation was followed by HPLC under multiple turnover conditions in which MCR\textsubscript{red1} was incubated with a 100-fold excess of BPS (Fig. 7). MCR\textsubscript{red1} acts as a catalyst in this reaction, since the amount of propanesulfonate formed is 11-fold greater than the amount of enzyme. There are two major differences between the results of the steady-state reaction (Fig. 7) and the single turnover reaction (Fig. 6). The \( k_{\text{obs}} \) for the single turnover reaction (2.6 s\(^{-1}\)) (Fig. 6), which equals the rate constant for formation of MCR\textsubscript{PS} (Figs. 4 and 5), is much faster than the steady-state rate (2.2 h\(^{-1}\), calculated based on either the exponential \( k_{\text{obs}} \) or the initial velocity (linear portion) in Fig. 7) of propanesulfonate formation, indicating that it is not chemistry but rather regeneration of MCR\textsubscript{red1} or propanesulfonate release that is rate-limiting in the steady-state reaction. In addition, in the single turnover reaction, BPS is fully converted to propanesulfonic acid, whereas in the steady-state reaction, only 10% conversion occurs. This indicates that MCR\textsubscript{red1} or MCR\textsubscript{PS} undergoes slow inactivation by conversion to a Ni(II) MCR-silent state during the steady-state reaction. Thus, as shown in Scheme 1, MCR\textsubscript{PS} can suffer two alternative fates: reactivation to form MCR\textsubscript{red1} or inactivation to form a Ni(II) silent state (or potentially a Ni(III) state). Furthermore, comparison of the steady-state and transient kinetics experiments provides strong evidence that the MCR\textsubscript{PS} state is a catalytically competent intermediate in propanesulfonate formation.

Reaction of MCR with BPS and Reversibility of MCR\textsubscript{PS} Formation—The catalytic conversion of BPS to propanesulfonate requires that the active MCR\textsubscript{red1} state be regenerated from MCR\textsubscript{PS} at each catalytic cycle (Scheme I). Ti(III) citrate was capable of supporting the steady-state reaction, indicating that reductive activation is required for the cycling of MCR between the MCR\textsubscript{PS} and MCR\textsubscript{red1} states.

Since HSCoB can donate electrons for methane formation from methyl-SCoM, we tested whether HSCoB could also regenerate MCR\textsubscript{red1} from MCR\textsubscript{PS}. In methane formation, the product is CoBSSCoM. In this case, we would expect to form CoBS-PS. However, HSCoB was unable to reconvert MCR\textsubscript{PS} to MCR\textsubscript{red1}. On the other hand, various small thioclates, including CoMSH, did convert MCR\textsubscript{PS} to MCR\textsubscript{red1}. To better understand...
the reaction of MCR\textsubscript{ps} with HSCoM, we monitored the reaction by EPR (Fig. S1) and UV-visible spectroscopy (Fig. 8). Conversion of MCR\textsubscript{ps} to MCR\textsubscript{red1} in the presence of HSCoM was determined by following the decrease of MCR\textsubscript{ps} at 420 nm and increase of MCR\textsubscript{red1} at 385 nm. At HSCoM concentrations between 0.2 and 4 mM, ~80% conversion of MCR\textsubscript{ps} to MCR\textsubscript{red1} was achieved (data not shown). The remaining 20% of MCR\textsubscript{ps} probably decays into MCR\textsubscript{silent}, which is indistinguishable from MCR\textsubscript{ps} by absorption spectroscopy but is EPR-silent. The rate

FIGURE 6. Rapid chemical quench studies of the reaction of MCR\textsubscript{red1} with BPS. A solution containing 90 \( \mu \)M MCR\textsubscript{red1} (in 50 mM Tris-HCl buffer, pH 7.6) was rapidly mixed with 300 \( \mu \)M BPS (150 \( \mu \)M, final) 50 mM Tris-HCl buffer, pH 7.6) at 20 °C. The decay of BPS (solid circle) and the formation of PS (open circle) were monitored by using HPLC connected to a conductivity detector. The rate constants, 2.6 ± 0.6 s\(^{-1}\) for BPS decay and 2.0 ± 0.6 s\(^{-1}\) for PS formation and the amplitudes 19.5 ± 1.7 nmol (39.7 ± 3.4 \( \mu \)mol) and 21.6 ± 2.9 nmol (44.0 ± 5.8 \( \mu \)mol) for BPS and for PS, were determined by fitting the data to single-exponential equations.
also is what led to the earlier assignment of BPS as an irreversible inhibitor. The slow rate of reactivation of MCR\textsubscript{ps} to MCR\textsubscript{red1} also indicates that the steady-state rate of PS formation will be very slow (as is shown below), because the multiple turnover reaction will be limited by the reactivation rate constant.

Ti(III) citrate is less efficient than CoMSH in converting MCR\textsubscript{ps} to MCR\textsubscript{red1}. Within 10 min at pH 10, Ti(III) citrate converted only ~5% MCR\textsubscript{ps} to MCR\textsubscript{red1}, whereas ~83% MCR\textsubscript{ps} converted to an EPR-silent form of the enzyme, and ~12% remained as MCR\textsubscript{ps} (Fig. 9). At pH 7.6, MCR\textsubscript{ps} was unaffected by Ti(III) citrate over the same period of time (data not shown).

The HSCoM-dependent conversion of MCR\textsubscript{ps} to MCR\textsubscript{red1} is pH-dependent, showing no conversion at pH 7.2 (data not shown). In an experiment otherwise identical to the one described in Fig. 8, 4 mM MeSCoM was used in place of HSCoM; under these conditions, MCR\textsubscript{ps} was not reactivated to MCR\textsubscript{red1} (data not shown), demonstrating the importance of the thiol group of HSCoM for reactivation. A survey of thiols has not been performed; however, dithiothreitol and mercaptoethanol were found to reactivate MCR\textsubscript{ps}.

As summarized in Schemes 1 and 2, these results indicate that BPS is not a competitive inhibitor or irreversible inactivator but rather a reversible redox inactivator of MCR (reversible by HSCoM) that can serve as an alternative substrate and that MCR\textsubscript{ps} is a more oxidized state than MCR\textsubscript{red1}, which can be converted back to the MCR\textsubscript{red1} state by thiolate nucleophiles or, less efficiently, by Ti(III) citrate.

**DISCUSSION**

BPS has been described as a highly potent competitive inhibitor (apparent $K_I = 50\ \text{nM}$) (41) and as an irreversible inhibitor (44) of MCR, and its high affinity has been exploited to titrate the active sites of this enzyme (11, 47). Here we clarify the reaction of the active MCR\textsubscript{red1} state with BPS by a combination of spectroscopic and kinetic studies. As shown in Schemes 1 and 2, this reaction occurs by the nucleophilic attack of Ni(I) on BPS to displace bromide and generate the EPR-active MCR\textsubscript{ps} species. MCR\textsubscript{ps} (formerly called MCR\textsubscript{BPS}) was described by Rospert et al. (28) and assigned as a high spin Ni(II)-alkylsulfonate radical species (29, 48). The lack of detectable hyperfine broadening from the halide of BPS (or any of the related compounds shown in Table 1 that give the MCR\textsubscript{ps} signal) provided evidence that the bromide undergoes elimination before or as the MCR\textsubscript{ps} state is formed. In fact, identical EPR spectra (MCR\textsubscript{ps}) are formed by incubating MCR\textsubscript{red1} with 3-bromo- ($I = 3/2$), and 3-iodo- ($I = 5/2$) propanesulfonate with no...
detectable hyperfine splitting from these halide atoms, indicating that the nickel is not positioned close to the halogen groups of these ligands (28). Advanced EPR studies have defined the hyperfine coupling constants between the Ni(III) center and the alkyl ligand; however, in this complex, 75% of the spin is in the nickel $d_z^2 - d_y$ orbital and ~7% on the attached methylene carbon atom (48). These values are in reasonable agreement with a DFT calculation on a methyl-Ni(III)–F₄⁺ model (49).

In the studies described here, MCRred₁ was reacted with BPS, and the propylsulfonate ligand was unambiguously identified (after acid quenching) as propanesulfonate by NMR spectroscopy and by HPLC analysis in both single and multiple turnover reactions. This experiment would not per se rule out the possibility that the various halopropane sulfonates ligate to nickel via the sulfonate oxygen(s), especially since similar MCRPS-like EPR spectra are generated when MCRred₁ is incubated with bromobutanesulfonate and chlorobutanesulfonate (Table 1). However, other sulfonates, including 3-mercaptop-1-propane-sulfonic acid, butanesulfonate, and propanesulfonate, do not induce MCRPS-like EPR signals. Furthermore, the reaction of MCRred₁ with 4-bromobutyric acid (29) or 4-chlorobutyric acid generates an EPR signal nearly identical to MCRPS. Therefore, the sulfonate group is not a strict requirement for generation of the MCRPS signal, only its anionic character. In addition, the advanced EPR studies described above strongly indicate the formation of an organometallic alkyl-nickel species (49).

Besides its intermediacy in PS formation from BPS, MCRPS is interesting in sharing some striking similarities to the MCRox₁ state. These two states are functionally similar in that they can be activated to the MCRred₁ state, however, it has so far proven impossible to activate other Ni(II) states of MCR. The “ready” nature of the MCRPS and MCRox₁ states must be related to their electronic structures, which have been assigned as Ni(II) associated with a radical, a thiol radical in the case of MCRox₁ (15, 17) and an alkylsulfonate radical for MCRPS (29). The UV-visible spectra of these two species are indistinguishable, indicating that MCRox₁ and MCRPS share the same nickel oxidation state. The EPR spectra are similar (for MCRox₁, the $g$ values are 2.231, 2.153, and for MCRPS, they are 2.223, 2.115), which would be consistent with the two species sharing the same oxidation state but different coordination states. Since the four planar nitrogens of the macrocycle are unlikely to vary, the spectral differences must result from changes in the upper axial ligands: a thiol radical for MCRox₁ versus an alkyl radical in the case of MCRPS.

In the presence of the low potential reductant Ti(III) citrate, the MCRPS state undergoes reductive activation and protonolysis to form MCRred₁ and propanesulfonic acid. Some thiolates like HSCoM, dithiothreitol, and mercaptoethanol also react with MCRps to regenerate MCRred₁ and presumably a thioether product, whose characterization is under way. Thus, BPS can serve as an alternative substrate for MCR. In these reactions, the rate of MCRPS formation ($k_{max}/K_m = 190$ mm$^{-1}$ s$^{-1}$, $k_{max}$ $= 17$ s$^{-1}$ at 20 °C) is ~60-fold faster than the rate of methane formation from the natural substrates, methyl-SCoM and HSCoB ($k_{cat}/K_m = 3$ mm$^{-1}$ s$^{-1}$ at 20 °C, 50 mm s$^{-1}$ at 60 °C (23)). However, the elimination of PS and regeneration of MCRred₁ occurs 1000-fold more slowly than methane formation from the natural substrates. The large difference between the rates of MCRPS formation and regeneration of MCRred₁ explains the nearly stoichiometric accumulation of MCRPS and rationalizes why BPS acts as such a strong inhibitor of MCR.

Schemes 1 and 2 rationalize the seemingly disparate properties of BPS, which have led to its classification as a competitive inhibitor (41), as an irreversible inhibitor (44), and, as described here, as an alternative substrate and a redox inactivator. Based on steady-state kinetics, BPS would appear to be a competitive inhibitor, because the active enzyme (MCRred₁) can react either with BPS or methyl-SCoM and because there is a path from MCRPS back to active MCRred₁. Therefore, in comparing the competitive inhibition Mechanism A with the redox inactivation/reactivation Mechanism B (Scheme 2), as long as the reaction of MCRred₁ with BPS is rapid and complete (i.e., the enzyme rapidly and quantitatively returns to its active state), one would not suspect the occurrence of a series of complex underlying reactions. However, by following enzyme-monitored turnover reactions, it is clear that BPS is not a traditional competitive inhibitor in a simple rapid equilibrium with free enzyme nor an irreversible inactivator, because there is a redox pathway back to the active enzyme form.

These results demonstrate conclusively that the MCRPS state is a catalytically competent intermediate in propanesulfonate formation and constitute the first spectroscopic observation of any intermediates in the MCR reaction that can be related to catalysis. The results could thus be considered to support the intermediacy of an alkynickel intermediate in MCR-catalyzed methane formation from methyl-SCoM. In the rest of this discussion, the results are discussed in relation to Mechanisms I and II.

With respect to Mechanism I, the formation of the MCRPS intermediate at rates exceeding the rate of methane formation from methyl-SCoM indicates the possibility that an alkynickel intermediate is involved in methanogenesis. Furthermore, any reaction mechanism must exhibit the property of microreversibility; therefore, as shown in Fig. 11, our results provide evidence for an alkynickel intermediate in the final step of anaerobic methane oxidation. According to Mechanism I (Fig. 11A), this step involves the thiolytic cleavage of an alkynickel bond to
form the thioether, methyl-SCoM, and regenerate MCR$_{red1}$. Then proton transfer to the methyl group to form methane, followed by electron transfer to Ni(III) to generate Ni(II), would generate a disulfide anion radical, which is considered to be sufficiently reducing to convert Ni(II) back to the active MCR$_{red1}$ state.

On the other hand, the relationship of the alkynickel intermediate to Mechanism I deserves a few words of caution, since the properties of methyl-SCoM and BPS are quite different. Although the strength of the CH$_3$–SCoM bond (293 kJ/mol) is similar to that of the C–Br bond in BPS (~270 kJ/mol), neither the methyl group (if Ni(I) attacked the thioether sulfur) nor the mercaptoalkylsulfonate group (CoMS$^-$) (if nickel attacked the methyl group) of methyl-SCoM is a good leaving group. However, bromide is an excellent leaving group. The standard measure of the goodness of a leaving group is the $pK_a$ of its conjugate acid (HBr versus CH$_4$); the lower the $pK_a$, the better the leaving group ability. Methane is one of the weakest acids known, with a $pK_a$ of 50, whereas HBr is a very strong acid, with a $pK_a$ of ~9. HSCoM would have an intermediate $pK_a$ of around 8. Therefore, an ionic reaction of MCR$_{red1}$ with BPS to eliminate bromide and form Ni(III)-alkylsulfonate is quite reasonable; however, it is unlikely that Ni(I) reacts with methyl-SCoM to eliminate a methyl anion and form Ni(III)-SCoM or to eliminate $\text{SCoM}$ and form methyl-Ni(III). Furthermore, computational results suggest that cleavage of a high energy methyl-SCoM (~70 kcal/mol) bond to form a weak methyl-nickel (less than 25 kcal/mol) bond is thermodynamically unreasonable, and a radical mechanism (Fig. 11C) was proposed that involves reaction of Ni(I) with the sulfur of methyl-SCoM to generate a Ni(II)-thiolate and a methyl radical, which abstracts a hydrogen atom from HSCoB (27, 50).

The role of HSCoB in the MCR reaction was assessed by single turnover kinetic studies using the chemical quench technique with radioactive [14C-methyl]SCoM as the substrate. These transient kinetic studies demonstrated that formation of even a single equivalent of methane requires HSCoB (23). This reaction was repeated with an analog of HSCoB that is one carbon shorter in length (mercaptoHEXanoyl-threonine phosphate). A single exponential decay was observed in both reactions that depended on the concentration on HSCoB or the analog, which was shown to react 1000-fold slower than HSCoB. These results indicate that HSCoB is not required for proton donation, since the acidic conditions of the quench would have supplanted that role by protonolysis of the methyl-nickel intermediate to form methane and indicate that HSCoB has an integral role in a step(s) that promotes C–S bond cleavage. Conversely, with respect to the methyl radical mechanism, the results do not rule out a requirement for HSCoB in hydrogen atom abstraction or in promoting a conformational change that might alter the reaction pathway and/or the regiospecificity of the attack of Ni(I) on methyl-SCoM.
HSCoM is firmly held in the active site of MCR by interactions of the thiol group with nickel, one of the sulfonate oxygens with a backbone nitrogen in the α subunit, and another sulfonate oxygen with Arg-120 of the γ subunit (Fig. 10). The interactions of the sulfonate oxygens greatly influence the type of substrate that can react with MCR. When the structures of the various sulfonate and carboxylate analogs shown in Table 1 are compared, it appears that generation of the MCRPS signal requires a good leaving group adjacent to an electrophilic carbon atom that is four bonds (∼4.8 Å) from the negatively charged oxygen of a carboxylate or sulfonate. When this distance is increased to ∼6 Å with BBS, the second order rate constant for reaction of MCR_{red}, with the bromoalkanesulfonate decreases by 70-fold. Interestingly, the electrophilic carbon in BPS is in the same position as the thioether sulfur of methyl-SCoM and the bromine group of BPS is located at approximately the same position as the methyl group of methyl-SCoM. These results indicate that, for the natural substrate, steric considerations would favor attack of Ni(I) at the sulfur of methyl-SCoM. However, as mentioned above, it is possible that binding of HSCoB could alter the stereoselectivity of the active site.

By strict analogy with the BPS reaction and as shown in Fig. 11B, in which attack of Ni(I) on C-3 of BPS followed by elimination of the bromide would generate the MCRPS state, one might expect Ni(I) to react with the thioether sulfur of methyl-SCoM to generate a Ni(III)-thiolate and eliminate a methyl anion. However, the poor leaving group properties of a methyl group as discussed above would prevent such a reaction. On the other hand, the attack of Ni(I) on the thioether sulfur of methyl-SCoM to form a Ni(III)-thiolate and release a methyl radical, as described in Fig. 11C, would retain the steric constraints of the reaction, based on the substrate profile described in the legend to Fig. 1. This step, leading to the formation of a relatively strong Ni(III)-thiolate bond and release of a methyl radical, was proposed to be feasible, with a barrier of ∼17–20 kcal/mol (instead of ∼45 kcal/mol for generation of the methyl-Ni) (27). Hydrogen atom abstraction from HSCoB by the methyl radical to form methane was predicted to have a very small barrier of ∼1 kcal/mol (27).

As shown in Fig. 11C, the reaction of MCR_{PS} with a thiolate to form the thioether and regenerate MCR_{red} is similar to the proposed reaction of the CoBS radical with the Ni(III)-thiolate to generate the disulfide anion radical and a Ni(II) form of MCR (Fig. 11C). Electron transfer from the disulfide anion radical to Ni(II) would form the disulfide and Ni(I)-MCR_{red}. Interestingly, in methane formation, cleavage of the C–S bond of methyl-SCoM appears to be rate-limiting, whereas in the BPS reaction, the first step (elimination of bromide) is rapid, and regeneration of MCR_{red} is rate-limiting.

In conclusion, the results described here are relevant to the initial step in methane formation and to the final step in anaerobic methane oxidation. Further studies involving analogs of methyl-SCoM and HSCoB are under way to uncover details of the MCR catalytic mechanism and to discriminate between the two proposed mechanisms for this unique nickel enzyme.

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