J|A|C|S
ARTICLES
Published on Web 02/04/2003

Rapid Ligand Exchange in the MCRred1 Form of Methyl-coenzyme M Reductase

Kuljeet Singh, Yih-Chern Horng, and Stephen W. Ragsdale*

Contribution from the Department of Biochemistry, Beadle Center, 19th and Vine Streets, University of Nebraska, Lincoln, Nebraska 68588-0664

Received September 12, 2002; Revised Manuscript Received November 27, 2002; E-mail: sragsdale1@unl.edu; ksingh@unlserve.unl.edu; yhorng@unlserve.unl.edu

Abstract: Methyl-coenzyme M reductase (MCR) from *Methanothermobacter marburgensis (Mtm),* catalyses the final step in methane synthesis in all methanogenic organisms. Methane is produced by coenzyme B-dependent two-electron reduction of methyl-coenzyme M. At the active site of MCR is the corphin cofactor F_{430}, which provides four-coordination through the pyrrole nitrogens to a central Ni ion in all states of the enzyme. The important MCRox1 ("ready") and MCRred1 ("active") states contain six-coordinate Ni(I) and differ in their upper axial ligands; furthermore, red1 appears to be two-electrons more reduced than in ox1 and other Ni(II) states that have been studied. On the basis of the reactivity of MCRred1 and MCRox1 with a substrate analogue and inhibitor (3-bromopropanesulfonate) and other small molecules (chloroform, dichloromethane, mercaptoethanol, and nitric oxide), we present evidence that the six-coordinate Ni(I) centers in the MCRred1 and MCRox1 states exhibit markedly different inherent reactivities. MCRred1 reacts faster with chloroform (2100-fold or 35000-fold when corrected for temperature effects), nitric oxide (90-fold), and 3-bromopropanesulfonate (10^6-fold) than MCRox1. MCRred1 reacts with chloroform and dichloromethane and, like F_{430}, can catalyze dehalogenation reactions and produce lower halogenated products. We conclude that the enhanced reactivity of MCRred1 is due to the replacement of a relatively exchange-inert thiol ligand in MCRox1 with a weakly coordinating upper axial ligand in red1 that can be easily replaced by incoming ligands.

Introduction

Methane, the byproduct of energy metabolism by methanogenic microbes, is a potent greenhouse gas that is produced in most anaerobic environments at annual global levels of 10^9 tons.[1] The nickel enzyme, methyl-coenzyme M reductase (MCR) catalyses the final step in methane synthesis (reaction 1) by methanogenic microbes.[1,2] Synthesis of methane is achieved by two-electron reduction of methyl-coenzyme M (methyl-SCoM, 2-(methylthio)ethanesulfonate) by coenzyme B (CoBSH, 7-thioheptanoylthreonine phosphate) to produce the mixed disulfide of CoBSH and CoMSH, CoBSSCoM.

$$\mathrm{MeSCoM} + \mathrm{CoBSH} \rightleftharpoons \mathrm{CH_4} + \mathrm{CoBSSCoM} \qquad \Delta G = -45\ \mathrm{kJ\ mol^{-1}} \quad (1)$$

MCR is a 300 kDa dimer of three different subunits $(\alpha\beta\gamma)_2$ containing one equivalent of coenzyme F_{430}, a nickel-tetrapyrrole, in each α subunit. The MCR from *Methanothermobacter marburgensis (Mtm)* has been most extensively studied. Several EPR-active forms of the enzyme are known (Figure 1): MCRred1, MCRred2, MCRox1, and MCRox2.[3] In addition, the EPR-silent MCR-silent, MCRred1-silent, and MCRox1-silent forms have been characterized.[1] The active MCRred1 state is formed in vitro by reducing the MCRox1 form with Ti(III)

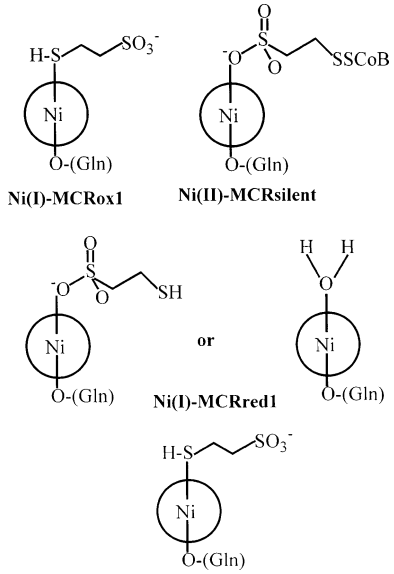
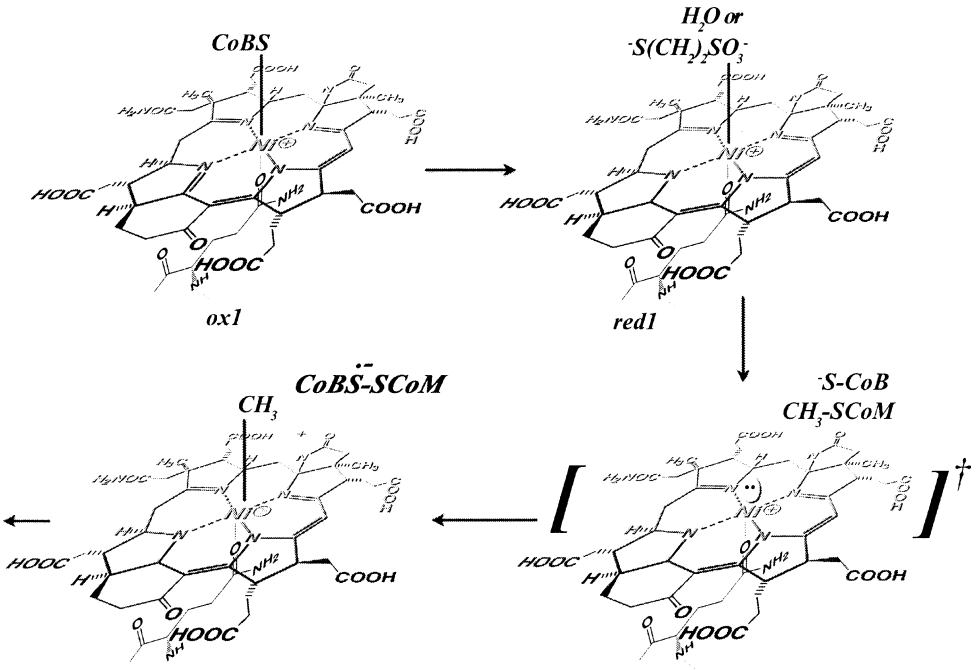


Figure 1. Models for the various states of MCR. All silent forms contain Ni(II), whereas the "ready state" (MCRox1) and "active state" (MCRred1) contain Ni(I). Only silent states shown here are characterized by X-ray spectroscopy. The circle around nickel represents the tetrapyrrole ring of F_{430}.

(1) Thauer, R. *Anton Leeuwenhoek Int. J. Gen. Microbiol.* **1997**, *71*, 21−32.
(2) Ragsdale, S. W. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: New York, 2002; Vol. 2.
(3) Mahlert, F.; Grabarse, W.; Kahnt, J.; Thauer, R. K.; Duin, E. C. *J. Biol. Inorg. Chem.* **2002**, *7*, 101−112.

10.1021/ja028525+ CCC: $25.00

Scheme 1



citrate at pH 10 and at 60 °C,[3−6] or in vivo by treating the cells with 100% H_2 before harvesting.[7] The MCRox1 state is generated by switching the gas phase of growing cells from H_2/CO_2 to N_2/CO_2 [8] or by adding sodium sulfide to the medium just before harvesting.[6] Without these treatments, the enzyme is isolated in an inactive Ni(II) EPR-silent state.

The Ni(II) states are well-characterized by X-ray crystallography at very high resolution;[9−11] however, the crystal structures of the catalytically important, but extremely labile, states such as MCRred1 (active state) and MCRox1 ("ready state"), have not been determined. Structural information about the Ni site has been gleaned from spectroscopic studies. In the red1-silent, ox1-silent, and silent forms of MCR, the Ni active sites are six-coordinate with four tetrapyrrole nitrogen ligands in the plane, the carbonyl oxygen of a side-chain glutamine in the lower axial position, and a variable upper axial ligand (see Scheme 1).[9,10] In the red1-silent and ox1-silent states, the thiolate of $CoMS^-$ is the upper ligand, whereas, in MCR-silent, a sulfonate oxygen from the CoMSSCoB disulfide is the upper axial ligand.

What is the coordination state of the active form of MCR? This is important because a methyl-nickel intermediate has been proposed in the mechanism for methane formation.[12,13] The methyl-nickel hypothesis is based predominantly on model chemistry. The Ni(I) form of the pentamethyl ester of F_{430} reacts with activated methyl donors (e.g., methyl iodide, methyl tosylate, methyl dialkyl sulfonium) to yield methane by protonation of a methyl-nickel intermediate.[14−16] Additionally, a radical pair, consisting of the Ni(I) state of F_{430} and a thiyl radical, can react with a methyl thioether to yield methane and the corresponding disulfide.[17]

Information on the coordination states of the Ni(I) forms of MCR is based on spectroscopy. EPR, ENDOR, resonance Raman, and X-ray absorption studies demonstrate that both the ox1 and red1 are Ni(I) states.[7,18−20] Surprisingly, XAS results indicate that the Ni(I) sites in both MCRred1 and MCRox1 are six-coordinate.[20] A six-coordinate Ni(I) ligand environment is also consistent with the results of studies in which the structurally characterized MCRox1-silent and MCR-silent forms were cryoreduced and analyzed by EPR and ENDOR spectroscopy.[19] When these MCR forms are reduced at low temperatures that restrict ligand rearrangements (77 K), the ox1-silent (S in the sixth coordination site) and silent (O in the sixth position) states convert to states with spectroscopic properties resembling those of ox1 and red1, respectively. Upon annealing at higher temperatures, they rearrange more quantitatively to these states. Axial ligation of the substrate to Ni(I) also has been suggested on the basis of enhanced spectral resolution of the ^{14}N-superhyperfine lines in the red1 EPR spectrum when methyl-CoM is added to the red1 form of the enzyme.[3] The results of XAS spectroscopy also indicate that the ox1 and red1 states are six-coordinate.[20] XAS, UV−visible, and Raman spectroscopy coupled with reductive titrations also indicate that reductive

(4) Goubeaud, M.; Schreiner, G.; Thauer, R. K. *Eur. J. Biochem.* **1997**, *243*, 110−114.
(5) Mahlert, F.; Bauer, C.; Jaun, B.; Thauer, R. K.; Duin, E. C. *J. Biol. Inorg. Chem.* **2002**, *7*, 500−513.
(6) Becker, D. F.; Ragsdale, S. W. *Biochemistry* **1998**, *37*, 2639−2647.
(7) Albracht, S. P. J.; Ankel-Fuchs, D.; Böcher, R.; Ellermann, J.; Moll, J.; van der Zwann, J. W.; Thauer, R. K. *Biochim. Biophys. Acta* **1988**, *941*, 86−102.
(8) Rospert, S.; Böcher, R.; Albracht, S. P. J.; Thauer, R. K. *FEBS Lett.* **1991**, *291*, 371−375.
(9) Grabarse, W. G.; Mahlert, F.; Duin, E. C.; Goubeaud, M.; Shima, S.; Thauer, R. K.; Lamzin, V.; Ermler, U. *J. Mol. Biol.* **2001**, *309*, 315−330.
(10) Ermler, U.; Grabarse, W.; Shima, S.; Goubeaud, M.; Thauer, R. K. *Science* **1997**, *278*, 1457−1462.
(11) Grabarse, W. G.; Mahlert, F.; Shima, S.; Thauer, R. K.; Ermler, U. *J. Mol. Biol.* **2000**, *303*, 329−344.
(12) Thauer, R. K. *Microbiology (UK)* **1998**, *144*, 2377−2406.
(13) Berkessel, A. *Bioorg. Chem.* **1991**, *19*, 101−115.
(14) Lin, S.-K.; Jaun, B. *Helv. Chim. Acta* **1991**, *74*, 1725−1738.
(15) Lin, S.-K.; Jaun, B. *Helv. Chim. Acta* **1992**, *75*.
(16) Jaun, B.; Pfaltz, A. *J. Chem. Soc., Chem. Commun.* **1988**, *1327*, 293−294.
(17) Signor, L.; Knuppe, C.; Hug, R.; Schweizer, B.; Pfaltz, A.; Jaun, B. *Chem.−Eur. J.* **2000**, *6*, 3508−3516.
(18) Telser, J.; Horng, Y.-C.; Becker, D.; Hoffman, B.; Ragsdale, S. W. *J. Am. Chem. Soc.* **2000**, *122*, 182−183.
(19) Telser, J.; Davydov, R.; Horng, Y. C.; Ragsdale, S. W.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 5853−5860.
(20) Tang, Q.; Carrington, P. E.; Horng, Y.-C.; Maroney, M. J.; Ragsdale, S. W.; Bocian, D. F. *J. Am. Chem. Soc.* **2002**, *124*, 13242−13256.

activation of ox1 to red1 involves two-electron reduction of a C=N bond in ring B or D of the F_{430} macrocycle.[20]

If methyl-Ni is an intermediate, a six-coordinate Ni(I) state for MCRred1 is counterintuitive since an open upper axial coordination site would be poised for reaction with the methyl group of methyl-SCoM (Scheme 1). Thus, how does MCR generate a methyl-Ni intermediate when the upper axial Ni(I) site is occupied? A related question is: why must six-coordinate Ni(I) ox1 be converted to six-coordinate Ni(I) red1 to exhibit activity? How do reduction of the F430 macrocycle and the ligand switch between S (ox1) and O (red1) lead to activation? The hypothesis explored here is that the ligand switch positions a kinetically more labile ligand in the upper axial site. This could promote interaction with the methyl group of methyl-CoM through an associative or dissociative ionic mechanism or through a radical mechanism. Structural studies can provide important insights into the ground states of a reaction; here the focus is on kinetic studies to evaluate the exchange lability of the ligands in the ox1 and red1 states. The results support the hypothesis that enhanced activity of the red1 state is significantly derived from increased ligand exchange reactivity of the upper axial Ni ligand.

Experimental Details

Materials. *M. marburgensis (Mtm)* was obtained from the Oregon Collection of Methanogens catalogued as OCM82. Chloroform, dichloromethane, iodomethane, ethanol, and thioethanol were purchased from Aldrich. Sodium sulfite and all buffers, media components, and other reagents were purchased from Sigma-Aldrich. All chemicals were used as purchased without further purification. Solutions were prepared in deionized water. Nitrogen (99.98%), N_2/H_2 (90%/10%), argon (99.8%), H_2/CO_2 (80%/20%), and CH_4/N_2 (0.2%/99.8%) were obtained from Linweld (Lincoln, NE). All halogenated substrates were prepared in ethanol, and fresh solutions were made immediately before use. Water-soluble substrates, such as thioethanol and 3-bromopropanesulfonate, were prepared in deionized water under strictly anaerobic conditions.

Preparation of Titanium Citrate Solution. A stock solution (200 mM) of titanium citrate was prepared by dissolving 0.75 g of titanium (III) chloride (Fluka) in 25 mL of 250 mM sodium citrate (Sigma-Aldrich) under anaerobic conditions. Then the pH was adjusted to neutrality by adding sodium bicarbonate from a saturated stock solution. The concentration of reducing equivalents was determined by titration with methyl viologen, which has a significantly higher redox potential than that of the Ti(III)/(IV) couple.

Growth, Cell Harvest, and MCR Purification. *M. marburgensis* cells were grown under $H_2/CO_2/H_2S$ (80%/20%/0.1%) at 65 °C in a 14-L fermentor as described.[4,21] Media was prepared as described earlier.[21] After 36 h ($A_{578} > 3.5$), 20 mM (final concentration) sodium sulfide was added to the growing cells prior to harvesting.[6] After incubating 15 min at 65 °C, the growing culture was cooled until the temperature fell to 25 °C. This procedure results in formation of MCRox1, which was purified by a known method, except that methyl coenzyme M was omitted from the lysis buffer.[4] The resulting purified MCRox1 was concentrated under argon using a 50-mL omega cell (Filtron) with a 30-kDa molecular mass cutoff.[22] Among different MCR preparations, 0.3−0.75 spins/mol of MCR_{ox1} per mole of Ni(I)-F_{430} was obtained. For reactivity studies with a particular molecule, such as chloroform, dichloromethane, and so forth, preparations with the same MCR_{ox1} spin integrations were used. The actual spin concentrations are given in the Figure captions.

Activation of MCR. The amounts of MCRox1 and MCRred1 were determined by EPR spectroscopy by comparing the double-integrated spin intensity with that of a 1 mM copper perchlorate standard. MCR was activated to the red1 state by adding titanium citrate at a molar ratio of 1:20 (ox1:Ti(III)) to a solution of MCRox1 in 0.5 M TAPS buffer, pH 10, and incubating at 65 °C for 45 min. Conversion to the MCRred1 state was assessed by measuring the double integrated intensity of the red1 and ox1 EPR signals. Among different activation experiments, 0.3−0.7 spin of MCRred1 per mole of Ni(I)-F_{430} was obtained and, for reactivity studies, enzyme solutions with similar spin samples were used. See the Figure captions for specific spin intensities.

Spectroscopy of MCR. Electron paramagnetic resonance (EPR) spectroscopy was done using Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller, a Hewlett-Packard model 5340 automatic frequency counter and Bruker gaussmeter. EPR spectra were recorded either at 77 or 100 K for a set of reactivity studies, and comparisons were made from spectra taken at same temperature. A liquid nitrogen coldfinger was used to record spectra at 77 K and helium gas was used to record the spectra at 100 K. The MCRox1 and MCRred1 spins were quantified using the following conditions: microwave power, 10 mW; temperature, 77 K; microwave frequency, 9.46 GHz; receiver gain, 20000; modulation amplitude, 12.8 G; modulation frequency, 100 kHz. A standard solution of copper perchlorate (1 mM) was used as the calibration standard for spin integrations. Solutions were prepared under strictly anaerobic conditions (Vacuum Atmospheres chamber, $O_2 < 10$ ppm). In each case, 200 μL of sample was used for the EPR studies. Reactions of MCRred1 with chloroform were performed at −20 °C using a junior laptop cooler (Sigma-Aldrich). The laptop cooler helps to keep the temperature at −20 °C for 1 h. Aliquots of MCRred1 (0.2 mL, 83 μM concentration, final) in 0.5 M TAPS buffer, pH 10, containing 20% glycerol were transferred to EPR tubes and placed in the −20 °C cooler. Then, prechilled $CHCl_3$ solutions (ethanolic solution of chloroform mixed with 0.5 M TAPS buffer and 20% glycerol, final concentration) were added using a Hamilton syringe with a long needle to begin each reaction in an EPR tube, which was quenched in liquid nitrogen at different time intervals. EPR spectra were recorded at 100 K.

Saturated nitric oxide solutions (1.59 M at 20 °C)[23] were prepared by bubbling nitrogen gas for 10 min over anaerobic water, followed by bubbling nitric oxide gas for 15 min. Nitric oxide gas was passed through a saturated KOH solution to remove any acidic impurities prior to bubbling through the desired solution.

Products of the MCRred1 reaction with chloroform (e.g., dichloromethane, methyl chloride, and methane) and with dichloromethane (e.g., methyl chloride and methane) were determined by gas chromatography (GC). We used a Varian 3700 gas chromatograph containing a column (6 mm × 2 m) of 80/120 carbopack B-DA/4% carbowax (Supelco) equipped with a flame ionization detector. The conditions were: column temperature, 60 °C; injector temperature, 140 °C; detector temperature, 140 °C; helium pressure, 20 psi (flow rate = 50 mL/min); H_2 pressure, 40 psi (flow rate = 100 mL/min); and air pressure, 20 psi (flow rate = 150 mL/min). Under these conditions, excellent separation of halogenated hydrocarbons and methane was obtained. The retention times were: methane, 45 s; methyl chloride, 2 min; dichloromethane, 12 min; chloroform, 13.5 min. For the reaction, 1-mL samples of MCRred1 were transferred to a 5-mL serum vial that was tightly capped by a rubber septum crimp-stopped with aluminum foil inside the anaerobic chamber. Aliquots of the halogenated solutions (prepared inside the chamber as described above) were transferred to the serum vials and, at specific reaction times, 1 mL (with chloroform) or 0.5 mL (with dichloromethane) of gas phase was removed by a Hamilton gastight syringe and injected into the GC. To assess the amount of substrate utilized and product formed, the peak areas were determined and

(21) Schönheit, P.; Moll, J.; Thauer, R. K. *Arch. Microbiol.* **1980**, *127*, 59−65.
(22) Horng, Y.-C.; Becker, D. F.; Ragsdale, S. W. *Biochemistry* **2001**, *40*, 12875−12885.
(23) Budavari, S., Ed. *The Merck Index*, 10th ed.; Merck & Co.: Rahway, NJ, 1983.

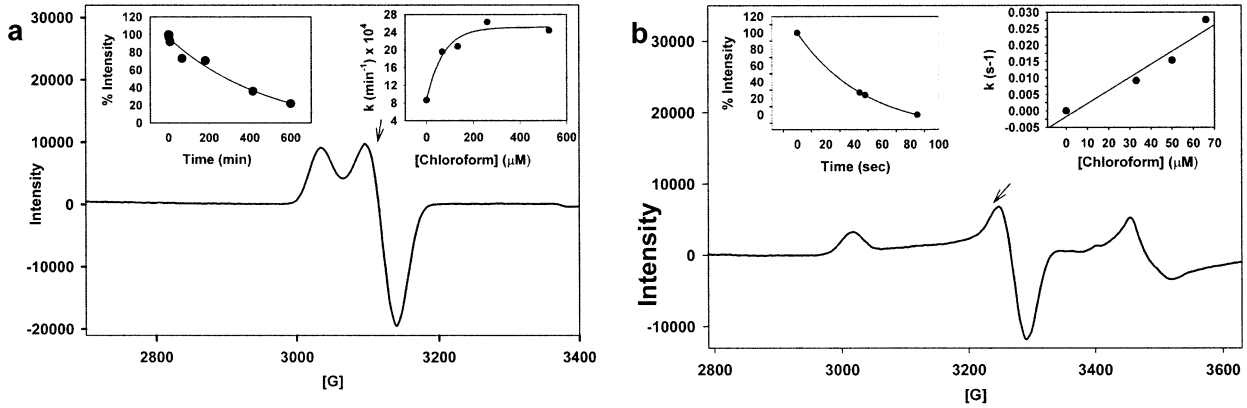


Figure 2. Reaction of MCR with chloroform. (a) First-order decay curve for the reaction of MCRox1 with chloroform at 25 °C. The reaction was monitored by following the decrease in EPR-signal intensity at $g = 2.160$. The MCRox1 concentration is 38 μM, the chloroform concentration is 66 μM, and the half-life for the decay is 354 min (left inset). The second-order rate constant was calculated by plotting first-order rates versus various chloroform concentration while keeping MCR concentration constant (right inset). The slope of tangent to the hyperbolic fit renders k_{on}. The arrow represents the field position at which MCRox1 decay was monitored. (b) First-order decay curve for the reaction of MCRred1 (42 μM) with chloroform (66 μM) at −20 °C. The calculated half-life for the decay is 33 s (left inset). The second-order rate constant was calculated by plotting first-order rate constants versus chloroform concentration (right inset). The slope of linear regression renders k_{on}. The arrow at $g = 2.060$ marks the field position at which MCRred1 decay was monitored.

Table 1. k_{on} Values for the Reaction of MCRox1 and MCRred1 with Various Substrates

MCR species	substrate	k_{on} ($M^{-1}s^{-1}$)[a]
ox1	$CHCl_3$	.15
red1	$CHCl_3$	3.2×10^2
ox1	NO	3.9
red1	NO	3.4×10^2
ox1	3-BPS	2.4
red1	3-BPS	3.4×10^6
ox1	2-thioethanol	0.58
ox1	CH_3I	0.3

[a] The second-order rate constants were calculated by plotting the first-order rate constants versus concentration at a constant MCR concentration. The slope of the tangent to the hyperbolic fit rendered k_{on}.

compared with standard curves that were generated prior to the reaction for each of the products.

Results

Table 1 summarizes all second-order rate constants utilized in these studies.

Reactions with Chloroform. The reactions of MCRox1 and MCRred1 with chloroform were monitored by measuring the decay of their characteristic EPR signals (Figure 2, a and b) and by GC analysis of the reaction products (Figure 3). The chloroform concentrations were varied over an 8-fold range from 66 to 525 μM. The MCRox1 EPR signal decayed slowly in an exponential manner (Figure 2, left inset), yielding a second-order rate constant at 25 °C of 0.153 M^{-1} s^{-1} (Figure 2, right inset). The natural decay rate of MCRox1, i.e., in the absence of chloroform, under these conditions is 8.6×10^{-4} min^{-1}, which is ~2-fold slower than the reaction with 66 μM chloroform. Even though chloroform quenches the MCRox1 EPR signal, we were unable to detect any products.

MCRred1 reacts quickly with chloroform, as observed previously.[8] Here we quantitatively compared the rates of reaction with ox1 and red1. Decay of the characteristic MCRred1 EPR signal at both 25 and 4 °C (Figure 2b) was too fast to monitor without resorting to rapid freeze quench conditions. However, it was possible to monitor the reaction at −20 °C in the presence of a cryosolvent (20% glycerol, final concentration) (Figure 2b, left inset), where a second-order rate

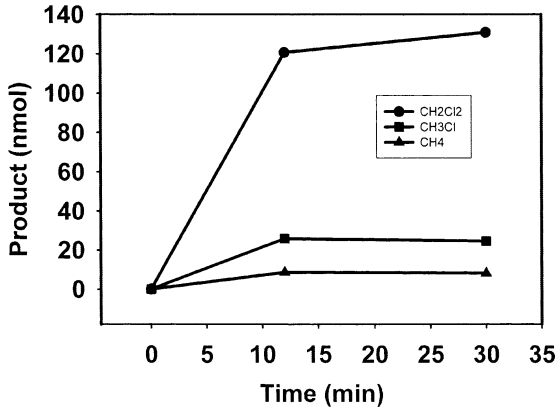


Figure 3. GC analysis of the reaction of MCRred1 with chloroform at 25 °C. MCR-red1 (110 nmol) was treated with 17 μmol of chloroform. A total of 165 nmol products were obtained (dichloromethane, 130 nmol; methyl chloride, 26 nmol; and methane, 9 nmol).

constant (k_{on}) at −20 °C of 3.21×10^2 M^{-1} s^{-1} was obtained by plotting the first-order rate constant versus chloroform concentration (Figure 2b, right inset). Thus, MCRred1 reacts over 2100-fold faster with chloroform than does MCRox1. Assuming a 2-fold increase in rate constant for each 10 °C increment in temperature, MCRred1 is predicted to react over 35000-fold faster than MCRox1 with chloroform. This result is in close agreement with the kinetic profile of MCRred1 and MCRox1 with 3-bromopropanesulfonate (vide infra).

Ni(I)-F_{430} reacts with chloroform to generate dichloromethane, methyl chloride, and methane.[24] Similarly, when the gas-phase of the MCR reaction was analyzed by GC, dichloromethane, methyl chloride, and methane were detected as products (Figure 3). With coenzyme F_{430} several turnovers were reported;[24] however, with the enzyme, apparently only one turnover was observed under these conditions. No dehalogenation products of chloroform (below 1% of the amount with the red1 state) were observed in control reactions performed in the absence of MCRred1 or in the presence of MCRred1-silent or MCRox1-silent. Previous work in our laboratory indicated that CoBSH

(24) Krone, U. E.; Laufer, K.; Thauer, R. K.; Hogenkamp, H. P. *Biochemistry* **1989**, *28*, 10061−10065.

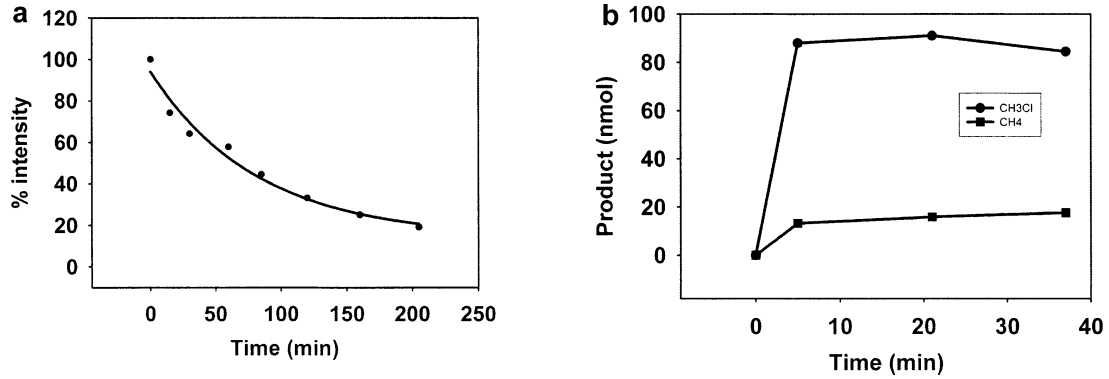


Figure 4. Reaction of MCR with dichloromethane. (a) Reaction of MCRox1 with dichloromethane (307 μM) at 25 °C. First-order decay of ox1 was monitored by following the decrease in the EPR-signal intensity at $g = 2.160$. The MCR concentration was 82 μM (MCRox1 was 33 μM), and the half-life for the decay was 57 min. (b) Reaction of MCRred1 with dichloromethane at 25 °C, monitored by GC. MCRred1 (81 nmol) was treated with 14 μmol of dichloromethane. A total of 107 nmol of products were obtained (91 nmol methyl chloride and 16 nmol methane).

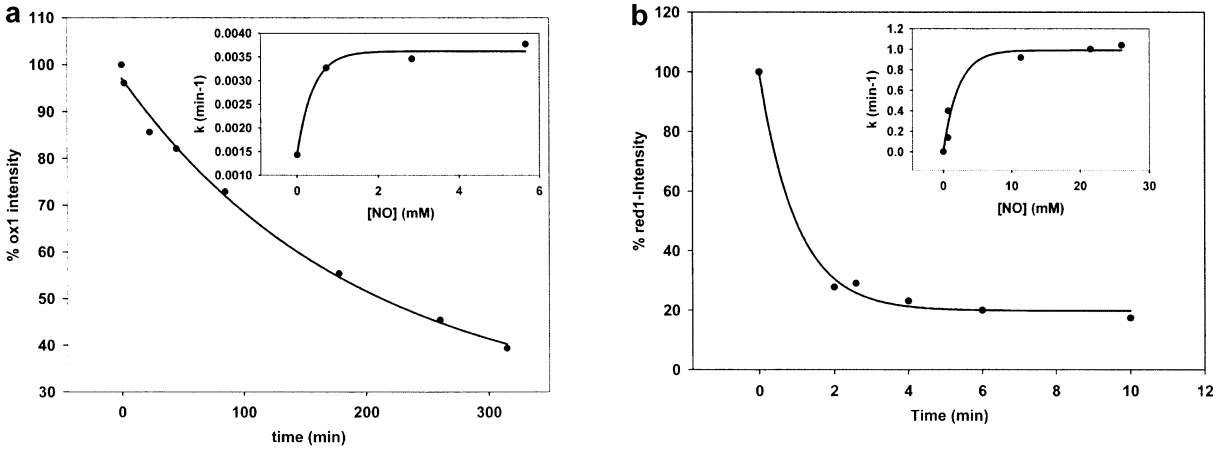


Figure 5. Reaction of MCR with nitric oxide (NO). (a) Reaction of MCRox1 with nitric oxide (NO) at 25 °C, monitored by decrease in EPR-signal intensity at $g = 2.160$. The MCR concentration was 212 μM ($MCR_{ox1} = 64$ μM), nitric oxide concentration was 722 μM, and the half-life for the decay is 212 min. The second-order rate was calculated by plotting first-order rate constants versus the concentration of NO (inset). The slope of the tangent to the hyperbolic fit renders k_{on}. (b) Reaction of MCRred1 with nitric oxide (NO) at 25 °C monitored by decrease in the EPR signal at $g = 2.060$. The MCR concentration was 210 μM (MCRred1 = 60 μM), the nitric oxide concentration was 21.5 mM, and the half-life for the decay was 0.69 min. The second-order rate constant was calculated by plotting first-order rates versus various chloroform concentration while keeping MCR concentration constant (inset). The slope of the tangent to the hyperbolic fit renders k_{on}.

is necessary for the formation of methane from methyl-SCoM, even under single-turnover conditions.[22] However, CoBSH did not noticeably increase the rate of reaction with chloroform (data not shown), which is consistent with the inability to perform multiple turnovers. These results indicate that chloroform, like substrate, reacts quickly with Ni(I) in the red1 state, yet, unlike substrate, causes inactivation by subsequent chemistry. In summary, MCRred1 reacts at least 10^4-fold faster than MCRox1 with chloroform and among the different forms of MCR tested (ox1, ox1-silent, red1-silent) is the only state able to generate lower halogenated products and methane.

Reactions with Dichloromethane. In contrast to the reaction with chloroform, MCRox1 and MCRred1 react at similar rates with dichloromethane. The first-order decay of the MCRox1 (Figure 4a) and MCRred1 (not shown) EPR-signals are 1.0×10^{-2} min^{-1} and 8.58×10^{-3} min^{-1}, 12-fold and 6-fold faster, respectively, than their natural decay rates in the absence of substrate. Analysis of the reaction products of the reaction of MCRred1 (Figure 4b) with dichloromethane by GC show that, as with chloroform, dehalogenation occurs to produce methyl chloride and methane. Apparently a single turnover is sufficient to inactivate the enzyme, as with chloroform. This result also follows the reactivity pattern as Ni(I)-F_{430}, which dehalogenates dichloromethane and, at pH 10, produces the same products we observe with MCR, albeit with several turnovers.[24]

It would have been interesting to follow the reaction of MCRred1 with methyl iodide. However, Ti(III) citrate, which is required to generate and stabilize MCRred1, catalyzes reduction of methyl iodide to methane. On the other hand, we did not observe any products of the reaction of Ti(III) citrate with chloroform or dichloromethane in the absence of enzyme.

Reactions with Nitric Oxide (NO). NO also quenches the EPR signals of MCRox1 and MCRred1 in a concentration-dependent manner (Figure 5). The second-order rate constant, k_{on}, for MCRox1, obtained from the slope of the tangent to the hyperbolic fit (Figure 5a, inset), is 3.94 M^{-1} s^{-1}. This value is very similar to that observed with chloroform (above), perhaps indicating similar slow ligand exchange reactions. Like chloroform, NO reacts much more rapidly with MCRred1 than with MCRox1, with a k_{on} value of 3.44×10^2 M^{-1} s^{-1} (Figure 5b).

The 90-fold enhanced reactivity of MCRred1 with NO relative to that of MCRox1 indicates that these two states could have a different immediate coordinate environment and supports the

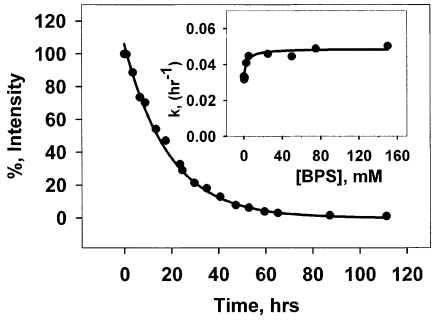


Figure 6. Reaction of MCRox1 with BPS. MCR (0.15 mM, MCRox1 = 78 μM) was mixed with 70 mM BPS in 0.5 M TAPS buffer (pH 10.0) at room temperature. The data were fit according to a single-exponential decay to yield a rate constant of 0.053 $\pm$ 0.002 h^{-1}. The inset shows BPS concentration dependence on MCRox1 decay. The data were fit with a hyperbolic binding equation, which gave a second-order rate constant of 2.44 M^{-1} s^{-1}.

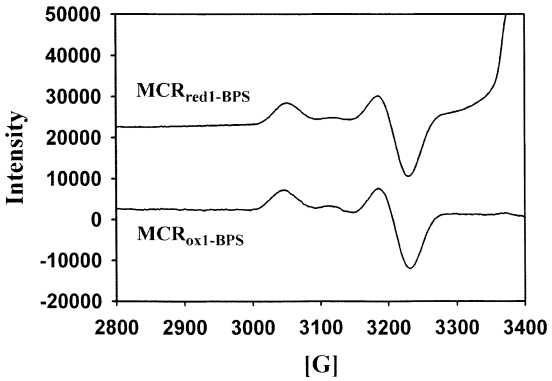


Figure 7. EPR spectra of MCRred1-BPS (53 μM) and MCRox1-BPS (52 μM). Both spectra were taken at 77 K in 0.5 M TAPS buffer, pH 10.0. The high-field ramp is the onset of the Ti(III) signal with all g values below 2. EPR conditions: microwave frequency, 9.48 GHz; modulation frequency, 100 kHz; modulation amplitude, 12.772 G; microwave power, 10 mW.

hypothesis that MCRox1 undergoes slow ligand exchange and MCRred1 exhibits fast ligand exchange.

Reactions with 3-Bromopropanesulfonate. We reacted MCR with 3-bromopropanesulfonate (3-BPS), which is a methyl-SCoM analogue and a potent competitive inhibitor of MCR [25] (Figure 6). With MCRox1, the decay rate is fairly slow ($k = 0.0534 \pm 0.0015$ h^{-1}); however, the reaction of BPS with MCRred1 occurs so quickly that it must be followed by rapid kinetics. On the basis of stopped-flow experiments, the BPS-induced decay of MCRred1 is found to be about 10^6-fold faster than that of MCRox1 under the same conditions (pH = 10, temperature = 25 °C, 0.5 M TAPS buffer). This extreme difference in reactivity between the two states of MCR must derive from a difference in active site accessibility or from a difference in inherent ligand exchange kinetics. Surprisingly, the EPR signals of MCR_{ox1} and MCR_{red1} after the reaction with BPS are indistinguishable (Figure 7).

We also reacted MCRred1 and MCRox1 with 2-mercaptoethanol (thioethanol), which is similar to CoMSH except that a hydroxy group replaces the sulfonate, When MCRox1 is treated with mercaptoethanol, the characteristic ox1 EPR signal (g = 2.16) decays about 15-fold faster than its natural decay rate (Figure 8). By varying the concentration of mercaptoethanol, a k_{on}, of 0.583 M^{-1} s^{-1} is obtained (Figure 8, inset). MCRred1

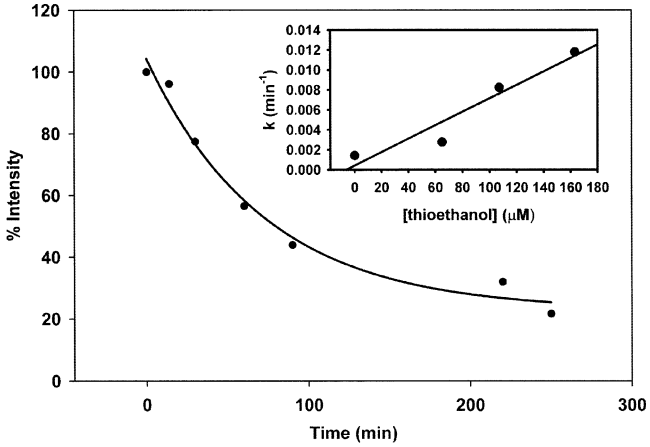


Figure 8. Reaction of MCRox1 with 2-mercaptoethanol at 25 °C. Decay of the MCRox1 EPR signal at g = 2.16 was monitored. The MCR concentration was 82 μM, the thioethanol concentration was 107 μM, and the half-life for the decay was 84 min. The second-order rate constant was calculated by plotting first-order rate constants versus NO concentration at constant MCR concentration (inset). The slope of linear regression renders k_{on}.

reacts with mercaptoethanol at a slightly faster rate ($k = 0.275$ min^{-1}), which is about 190-fold faster than the natural decay rate for MCRred1 (without mercaptoethanol) under these conditions (1.43×10^{-3} min^{-1}).

Discussion

Both MCRox1 and MCRred1 appear to contain Ni(I); however, only red1 is catalytically active. What is the origin of this enhanced activity? Our original hypothesis was that the resting state of the Ni(I) ion in MCRred1 would be four- or five-coordinate with an open upper axial site. This would allow the two nonbonding electrons in the d_{z^2} orbital to react with the methyl group of methyl-SCoM and form the putative methyl-Ni intermediate, which is almost universally accepted to be the direct precursor of methane (yet there are alternative proposals that do not involve methyl-Ni[26]). This would be analogous to the reaction of four-coordinate Co(I) in cobalamins with the methyl group of methyl-SCoM in the methyl-SCoM methyltransferases. (One difference is that Ni(I) is d^9, while Co(I) is d^8. To participate in an S_N2 reaction, dissociation of the sixth ligand during the transition state should be accompanied by an electronic rearrangement to put two electrons in the d_{z^2} orbital.) However, if both ox1 and red1 are six-coordinate, as indicated by XANES and EXAFS results,[20] the upper axial ligand must be replaced to form the alkyl-nickel intermediate. In a dissociative mechanism, a five-coordinate species would be an intermediate or transition state during the reaction. In an associative mechanism, a seven-coordinate system with two upper axial ligands could be formed in the transition state.[27] In either case, replacing a strong with a weak axial ligand could markedly accelerate the rate of ligand substitution. Perhaps, the two-electron reduction of the macrocycle also enhances the ligand substitution rate.

There is evidence that the upper axial ligand is different for the ox1 and red1 states (Scheme 1). XAS results indicate that ox1 contains a thiolate (presumably from CoM), while red1

(25) Ellermann, J.; Rospert, S.; Thauer, R. K.; Bokranz, M.; Klein, A.; Voges, M.; Berkessel, A. *Eur. J. Biochem.* **1989**, *184*, 63−68.
(26) Pelmenschikov, V.; Blomberg, M. R. A.; Siegbahn, P. E. M.; Crabtree, R. H. *J. Am. Chem. Soc.* **2002**, *124*, 4039−4049.
(27) Goldstein, S.; Czapski, G.; van Eldik, R.; Shaham, N.; Cohen, H.; Meyerstein, D. *Inorg. Chem.* **2001**, *40*, 4966−4970.

contains an oxygen donor (perhaps water or the sulfonate from methyl-SCoM) or nitrogen-donor ligand at this position. [20] If increased exchange lability is important in the activation, MCRred1 should exhibit enhanced reactivity toward small molecules that could diffuse into the active site and react with Ni(I). To test this hypothesis, we compared the rates at which the MCRox1 and MCRred1 forms of the enzyme react with small molecules with and without structural homology to methyl-SCoM.

Some of the compounds that were reacted with MCR quench or alter the Ni(I) EPR signal, and some also form products. Interestingly, nearly all the compounds tested quench the ox1 EPR signal at approximately the same slow rate, consistent with the notion that ligand exchange is rate-limiting in these reactions. MCRox1 appears incapable of reacting with methyl-SCoM to form methane.[4,6] Similarly, most react with MCRred1 significantly faster than with ox1, as predicted by the ligand-exchange hypothesis. The reaction rate for red1 with various compounds varies over a 10^6-fold range. The most reactive molecule with MCRred1 is BPS, a potent inhibitor and substrate analogue.[25,28] MCRred1 reacts with BPS over one million-fold faster than does MCRox1, yet, with both enzyme states, the reaction generates a distinct EPR signal, observed earlier,[28] that presumably arises from Ni(I). Decay of this new signal appears to be linked to debromination, forming propane sulfonate.[29] Except for mercaptoethanol, which resembles CoMSH, there is a slight apparent increase in the rate of reaction as the size of the compound decreases from chloroform, to dichloromethane, to methyl iodide, to NO. Presumably, the smaller molecules can enter the active site faster.

This enhanced reactivity with a substrate or substrate analogue could be argued to result from a substrate-induced conformational change, similar to that described upon binding CoM.[9] Part of the rate enhancement exhibited by red1 with BPS may indeed result from a more accessible active site. However, we tested several small molecules that do not resemble CoM or CoB, which would not be expected to induce a conformational change. For example, nitric oxide is a small diatomic molecule with no similarity to either MCR substrate. NO has been well-studied as an Fe ligand for heme and FeS clusters.[30−33] NO also is a radical that is expected to react with another radical, like Ni(I), to generate a diamagnetic product. Upon reaction with NO, decay of the EPR signal of MCR-red1 is 90-fold faster than with MCRox1. MCRred1 reacts over 35000-fold faster with chloroform than does MCRox1. As with BPS, dehalogenation occurs; however, like NO and unlike BPS, a stable intermediate EPR signal is not observed, and the ox1 or red1 EPR signals are quenched. These results indicate that Ni(I) is oxidized as chloroform undergoes dehalogenation.

The above results demonstrate that the Ni(I) ion in the red1 state is much more reactive than in the ox1 state. ENDOR[18] and XAS[20] results indicate that the four tetrapyrrole nitrogens and the lower axial O-donor ligand from glutamine remain fixed in the ox1 and red1 states; thus, the difference is in the upper axial ligand. Therefore, we presume that the first step in all these reactions (and possibly with substrate) is ligand exchange involving replacement of the upper axial ligand. Consequently, for example, when chloroform approaches the MCRox1 active site, it has to replace the CoMSH molecule before reacting with the nickel center to form the alkyl-Ni intermediate.

If the Ni−S bond is more stable than the Ni−C bond, the ligand exchange reaction would be unfavorable; if the relative transition states are assumed to follow energetic profiles that are similar to those of the ground states for the intermediates, the reaction would be very slow. On the basis of DFT calculations, the $RS-CH_3$ bond strength in methyl-SCoM is found to be ~70 kcal/mol, while the CH_3-Ni bond strength is ~25 kcal/mol.[26] Thus, the reaction of MCR (Ni−S) with methyl-SCoM to form methyl-Ni would be endothermic by 45 kcal/mol.[26] Predominantly on the basis of this mismatch, it was proposed that mechanisms involving formation of methyl-Ni are not feasible. However, a similar problem is faced in cobalamin-dependent methyltransferases. Similar to MCR, these reactions involve cleavage of high-energy methyl−N or methyl−S bonds. In these systems, a key step in the mechanism is electrophilic activation of the methyl group. For example, protonation of the nitrogen of tetrahydrofolate to which the methyl group is attached activates the methyl group toward nucleophilic attack by Co(I).[34] Thus, we do not agree that this bond strength argument necessarily rules out an alkyl-Ni intermediate since the relevant $RS-CH_3$ bond strength for the transfer of an activated methyl group from S to Ni could be significantly lowered, relative to that of the unactivated group.

Replacement of a more covalent Ni−S bond with a less stable ionic Ni−O bond would facilitate a ligand exchange reaction (Scheme 1). The strength of the Ni(II)−S bond in F430 is between 38 and 46 kcal/mol;[26] however the strength of a Ni(I)−S versus a Ni(I)−O is not known. Whether the ligand in the red1 state is water (or another weak axial ligand, perhaps the sulfonate group of the substrate/anologue/product) is an open question. As outlined in Scheme 1, in the MCRred1 state, the upper axial site would contain a weakly coordinating oxygen donating molecule. Chloroform would approach Ni(I) from the top of the planar ring and rapidly react with the nickel site by replacing the O-donor ligand (fast ligand exchange) (Scheme 1). Ligand replacement could occur through a dissociative mechanism involving a five-coordinate intermediate (as shown in Scheme 1) or an associative mechanism involving a seven-coordinate species. If ligand substitution by a dissociative mechanism were the rate-determining step, all of the substrates would react with red1 at the same (or similar) rate. This is not the case.The former mechanism is more common; however, recent work by Meyerstein on a variety of methyl transfer reactions suggests the possibility of an associative mechanism in which both the upper axial oxygen/nitrogen ligands remain as the methyl−Ni bond is formed.[35] In both scenarios, replacement of the strongly coordinating ligand enhances reactivity. With BPS, a fairly stable Ni(I) state is formed before debromination occurs. With NO, the signal is quenched immediately upon reaction.

(28) Rospert, S.; Voges, M.; Berkessel, A.; Albracht, S. P. J.; Thauer, R. K. *Eur. J. Biochem.* **1992**, *210*, 101−107.
(29) Horng, Y. C.; Ragsdale, S. W. Manuscript in preparation.
(30) Ding, H.; Demple, B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5146−5150.
(31) Sellers, V. M.; Johnson, M. K.; Dailey, H. A. *Biochemistry* **1996**, *35*, 2699−2704.
(32) Kennedy, M. C.; Antholine, W. E.; Beinert, H. *J. Biol. Chem.* **1997**, *272*, 20340−20347.
(33) Taoka, S.; West, M.; Banerjee, R. *Biochemistry* **1999**, *38*, 2738−2744.
(34) Seravalli, J.; Zhao, S.; Ragsdale, S. W. *Biochemistry* **1999**, *38*, 5728−5735.
(35) Van Eldik, R.; Meyerstein, D. *Acc. Chem. Res.* **2000**, *33*, 207−214.

As with NO, the reaction of MCRox1 or MCRred1 with chloroform leads to decay of the Ni(I) EPR signal and formation of an inactive Ni(II) state. GC analysis of the reaction demonstrates the formation of several dehalogenated products, such as, dichloromethane, methyl chloride, and methane. A mechanism can be proposed for the dehalogenation reactions (eqs 2−6). In the first step, (eq 2) attack of Ni(I)-F_{430} on $CHCl_3$ could generate a haloalkyl-nickel (III) intermediate, a reaction with chemical precedent.[36,37] This nickel (III) intermediate species could undergo one-electron reduction with Ti(III), which is present in the solution to generate MCRred1 from MCRox1, to generate a Ni(II)-alkyl (eq 3) or perhaps an unstable nickel (III) radical anion species (not shown). A radical anion species for cobalt has been previously proposed.[38] This unstable radical anion or the Ni(II)-alkyl could undergo either homolytic or heterolytic organometallic bond cleavage to generate a dichloromethyl radical (eq 4b), or dichloromethyl anion (eq 4a), respectively, that could abstract a proton to generate dichloromethane (eq 5). A similar series of reactions would explain the conversion of dichloromethane to methyl chloride and then to methane.

$$[\mathrm{Ni(I)}] + \mathrm{CHCl_3} \rightarrow [\mathrm{Ni(III)}-\mathrm{CHCl_2}] + \mathrm{Cl^-} \quad (2)$$

$$[\mathrm{Ni(III)}-\mathrm{CHCl_2}] + \mathrm{Ti(III)} \rightarrow [\mathrm{Ni(II)}-\mathrm{CHCl_2}] + \mathrm{Ti(IV)} \quad (3)$$

$$[\mathrm{Ni(II)}-\mathrm{CHCl_2}] \rightarrow \mathrm{CHCl_2^-} + [\mathrm{Ni(II)}] \quad \text{(heterolysis)} \quad (4a)$$

$$\rightarrow \bullet\mathrm{CHCl_2} + [\mathrm{Ni(I)}] \quad \text{(homolysis)} \quad (4b)$$

$$\mathrm{CHCl_2^-} + \mathrm{H^+} \rightarrow \mathrm{CH_2Cl_2} \quad (5)$$

We have observed only one turnover with the enzyme, suggesting that the Ni(II) product that is formed is unable to return to the catalytic cycle. Even addition of CoBSH, which is required for even a single turnover of methyl-SCoM,[22] does not lead to multiple turnovers. In support of this series of reactions, the reaction of MCRred1 with dichloromethane produces various dehalogenating products such as methyl chloride and methane. These results agree well with the reported dehalogenation activities of cofactor F_{430}.[24]

On the basis of studies of the volume of activation, van Eldik and Meyerstein proposed that the reaction of free radicals with metals or, in some cases, heterolysis of alkyl-metal complexes can be considered to be a ligand exchange reaction.[35] If this is true, based on the principle of microreversibility, the formation of a methyl-nickel(III) complex from Ni(I) could be considered to be a ligand exchange reaction. This work supports this concept for the MCR reaction. That this reaction can be classified as a ligand exchange reaction also supports the concept of a methyl-nickel intermediate in the MCR reaction mechanism.

The results indicate that the first step in the reaction of MCRred1 with its natural substrate is a ligand exchange reaction that places methyl-SCoM in the upper axial ligation site. Perhaps the substrate is folded back in a way that the methyl group is near the Ni or perhaps an equilibrium exists between oxygen and sulfur (CH_3-S) ligation. Another possibility, suggested by the finding that the tetrapyrrole ring in red1 is reduced by two electrons,[20] is that the reaction occurs by ligation of the sulfonate oxygen atom to Ni and electron/proton donation to the methyl group from the ring itself. These possibilities are under investigation.

Conclusions

Methanothermobacter marburgensis (formerly *Methanobacterium thermoautotrophicum*, strain Marburg) Δ*H* catalyzes the reductive dechlorination of 1,2-dichloroethane to ethylene and chloroethane by replacing two and one chlorines from the substrate molecule, respectively.[39] Factor F_{430} in solution can also dehalogenate CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl to lower dehalogenated hydrocarbons, all the way to methane.[24] Here we show that red1 form of MCR rapidly dehalogenates $CHCl_3$ and CH_2Cl_2 and related chlorinated and brominated compounds. On the basis of kinetic studies of the reaction of MCR with these and other small molecules including chloroform, NO, bromopropanesulfonate (BPS), and thioethanol, we have shown that the inherent reactivity of MCRox1 versus that of MCRred1 is quite different. A major component of this differential activity appears to be due to the rate at which the upper axial ligand undergoes ligand substitution, with the O-donor ligand in red1 being more exchange labile than ox1. The results indicate that the first step in the reaction of MCRred1 with its natural substrate is a ligand exchange reaction that places methyl-SCoM in the upper axial ligation site. In the case of substrates and substrate analogues (BPS), a conformational change could further increase the accessibility of the active site; however, chloroform and NO would not be expected to induce such a conformational change, and thus, we view the reactivity of MCR with these compounds to reflect inherent differences in ligand exchange lability between the two states.

Acknowledgment. This work was supported by DOE Grant (ER20053, S.W.R.).

JA028525+

(36) Scheffold, R.; Albrecht, S.; Orlin, R.; Ruf, H.; Stamouli, P.; Timebart, O.; Walder, L.; Weymuth, C. *Pure Appl. Chem.* **1987**, *59*, 363−377.
(37) Bekac, A.; Wspenson, J. H. *J. Am. Chem. Soc.* **1986**, *108*, 713−719.
(38) Krone, U. E.; Thauer, R. K.; Hogenkamp, H. P. C. *Biochemistry* **1989**, *28*, 4908−4914.
(39) Holliger, C.; Schraa, G.; Stupperich, E.; Stams, A. J.; Zehnder, A. J. *J. Bacteriol.* **1992**, *174*, 4427−4434.