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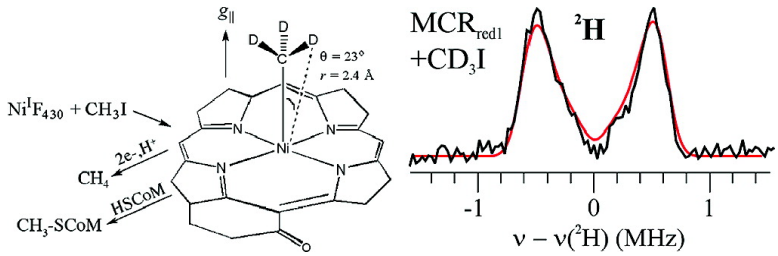

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Biochemical and Spectroscopic Studies of the Electronic Structure and Reactivity of a Methyl−Ni Species Formed on Methyl-Coenzyme M Reductase

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Methyl-coenzyme M reductase (MCR) catalyzes the formation of methane from the methylthioether methyl-coenzyme M and coenzyme B.[1] The active form of MCR, denoted MCR_{red1}, contains a nickel(I) F_{430}, tetrapyrrole.[2−4] Two mechanisms have been proposed for methane synthesis: one involves an organometallic methyl−Ni(III) intermediate,[5,6] while the other includes a methyl radical.[7] A methyl−Ni intermediate has never been trapped during the reaction of MCR with native substrates, although a propyl-3-sulfonate-Ni(III)F_{430} species has been characterized[8,9] and shown to undergo reactions resembling the proposed intermediate steps in methanogenesis and anaerobic methane oxidation.[10,11] The observation of a methyl−Ni MCR species is thus of considerable significance in terms of both biochemistry and bio-organometallic chemistry.

It was expected that the methyl−Ni(III) species formed during methanogenesis would be highly oxidizing and undergo immediate conversion to a methyl−Ni(II) state. Thus, it is surprising that reaction of MCR_{red1} with CH_3I converts the EPR spectrum of the former, $g_\perp = 2.065$, $g_{||} = 2.24$, to a stable axial signal, $g_\perp = 2.10$, $g_{||} = 2.22$, denoted MCR_{Me} (Figure 1; 35 GHz spectra, Figure S1, Supporting Information). The **g** tensors of both MCR_{red1} and MCR_{Me} centers surprisingly are characteristic of d^9 metal ions with the unpaired electron (hole) primarily localized in the $d_{x^2-y^2}$ orbital.

The rate of MCR_{Me} formation approximates the maximum rate of methane formation from methyl−SCoM and CoBSH (4.5 s^{-1} at 20 °C)[6] and is dependent on [CH_3I]; the second-order rate constant, 1900 M^{-1} s^{-1} (inset, Figure 1), is approximately 2-fold larger than the k_{cat}/K_M for methane formation from its natural substrates (930 M^{-1} s^{-1} at 20 °C and 1.9 × 10^4 M^{-1} s^{-1} at 65 °C).[6]

To establish the nature of the Ni center of MCR_{Me}, this state was prepared with both $^{13}CH_3I$ and CD_3I,[12] and we generated 2D field-frequency patterns comprising stochastic CW (^{13}C) or pulsed[13] ($^{1,2}H$) 35 GHz ENDOR spectra collected at multiple fields across the EPR envelope of the MCR_{Me} isotopologues, Figure 2.[14,15] In the frequency range, 0−27 MHz, Figure 2, the spectra of natural-abundance MCR_{Me} show ^{14}N ENDOR signals from the strongly coupled in-plane ^{14}N ligands of the F_{430} macrocycle. Analysis shows the hyperfine coupling is roughly isotropic, with $a(^{14}N) \approx 28$ MHz. The large value is characteristic of an in-plane ligand to a $S = {}^1/_2$ metal ion with its electron/hole in a $d_{x^2-y^2}$ orbital, as found for the parent MCR_{red1}, $a(^{14}N) \approx 29$ MHz,[16] a propyl-3-sulfonate-Ni product ($a(^{14}N) = 26.6-30.5$ MHz[8]), and MCR_{ox1} ($a(^{14}N) = 24.0-26.5$ MHz).[17]

At each field, the spectrum of $^{13}CH_3MCR_{Me}$ shows a single new peak, the ν_+ branch of a ^{13}C doublet with frequency, $\nu_+ = \nu_C + A(^{13}C)/2$. The value of ν_+ increases strongly with field as the field is lowered from $g_\perp$ to $g_{||}$, a dependence characteristic of an axial hyperfine tensor that is coaxial with **g**; simulations (Figure 2) yield the hyperfine tensor principal values, $A_{||} = a_{iso} + 2T = 43.0(5)$ MHz, $A_\perp = a_{iso} - T = 20.0(5)$, corresponding to $a_{iso} = 27.7$ MHz, $T = 7.7$ MHz. The presence of this large isotropic coupling unambiguously establishes that the CH_3 is covalently bound to the paramagnetic Ni(III) ion. At the same time, this coupling corresponds to only ∼1% spin density in a 2s orbital on ^{13}C.[18]

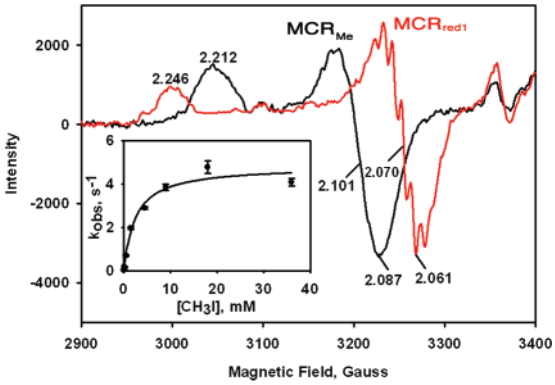

Figure 1. EPR spectrum of MCR_{Me} (black) formed by incubation of 10 μM MCR_{red1} (red) with 1 mM CH_3I in 50 mM Tris (pH 7.6) at 20 °C. The inset shows 10 μM MCR_{red1} converted to MCR_{Me} by reacting with varying concentrations of CH_3I in 50 mM Tris, pH 7.6 containing 0.2 mM Ti(III) citrate. The data were fit to a 2-parameter hyperbolic equation with k_{max} of 4.8 ± 0.3 s^{-1}, K_M of 2.5 ± 0.5 mM, (k_{max}/K_M of 1900 ± 400 M^{-1} s^{-1}).

Figure 3 presents the 2D field-frequency plot of $^2H(I = 1)$ stochastic Mims ENDOR spectra of CD_3MCR_{Me}. The ^2H spectra appear as doublets at frequencies, $\nu_\pm = \nu_D \pm A/2$, in which the further small[19] quadrupole splittings are not resolved. Simulation of the 2D ^2H pattern (Figure 3) shows that it is unlike the ^{13}C pattern (Figure 2) in that it is dominated by the dipolar contribution: $A_\perp = -0.58$, $A_{||} = 1.76$ MHz ($T = 0.78$ MHz, $a_{iso} = 0.2$ MHz), where the unique direction is at an angle, $\theta \approx 20°$ relative to $g_{||}$.[13,20]

The reaction of CH_3I with the Ni(I)F_{430} of MCR_{red1} thus is shown by EPR/ENDOR measurements to form a Ni−CH_3 bond through what is formally an oxidative addition to Ni(I):

$$[Ni^I(F_{430})] + CH_3I \rightarrow [CH_3-Ni^{III}(F_{430})]^+ + I^-$$

The puzzling observation that the Ni(I) ion of MCR_{red1} and the Ni(III) ion of MCR_{Me} both have $d_{x^2-y^2}$ odd-electron orbitals, even though the formal electron count on Ni differs by two, is understandable through a cartoon bonding scheme for a Ni−CH_3 fragment, Scheme 1, similar to one presented earlier.[23] This scheme shows the highest two d-orbitals of the parent Ni(I) center, occupied

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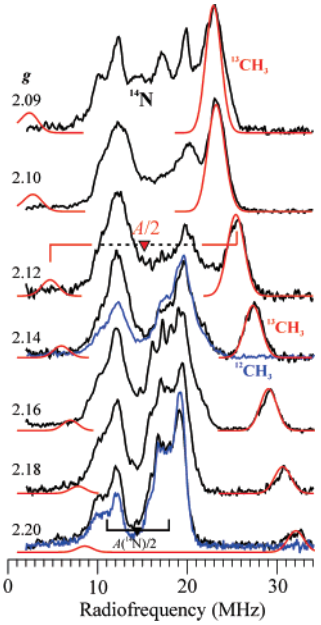


Figure 2. CW stochastic[21] 35 GHz ENDOR spectra of $^{13}CH_3MCR_{Me}$ (black) and $^{12}CH_3MCR_{Me}$ (blue; selected). The red traces are ^{13}C simulations using $g_\perp = 2.095$, $g_\parallel = 2.221$, $A_\perp = 20.0$, $A_\parallel = 43.0$ MHz, **A** collinear with **g**. "Goalposts" show $2\nu(^{13}C, ^{14}N)$; triangle indicates $A(^{13}C, ^{14}N)/2$. Conditions: 2 K; microwave frequency, 35.089 GHz; power ≈ 1 μW; 100 kHz field modulation amplitude, ~1.3 G; sample time, 1 ms; delay time, 1.5 ms; rf time, 2.0 ms, 200 scans.

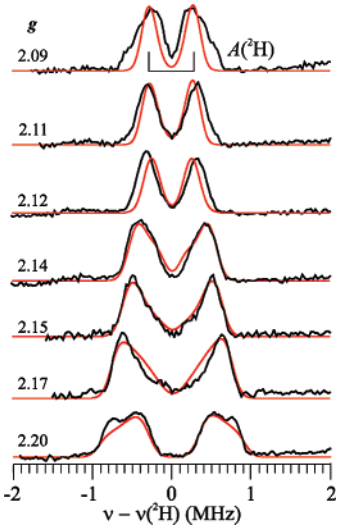


Figure 3. Mims stochastic 35 GHz ENDOR spectra for CD_3MCR_{Me}. Simulations (red): $A_\perp = -0.58$, $A_\parallel = 1.76$ MHz, $\theta = 18°$. Conditions: 2 K; frequency, 34.811 GHz; π/2-pulse, 50 ns; $\tau = 500$ ns; t_{rf}, 60 μs, with random selection ("hopping") of rf and baseline correction;[22] 20 scans.

by three electrons, and the empty sp^n hybrid of the hypothetical CH_3^+ with which this center reacts to form the Ni(III)-CH_3 fragment. The sp^n forms a strong two-electron bond with Ni d_{z^2} in which the bonding orbital primarily is associated with C, corresponding to a strong transfer of *charge* to C. The antibonding orbital lies above the $d_{x^2-y^2}$ orbital, unoccupied.

This picture explains why the odd-electron orbital is largely unperturbed by the oxidative addition reaction and why the transfer of *spin* to CH_3 is minimal: the Ni−C two-electron bond does not involve the odd electron, which in effect is a spectator to the reaction! The change in *g*-values can be assigned primarily to changes in the d-orbital splittings.

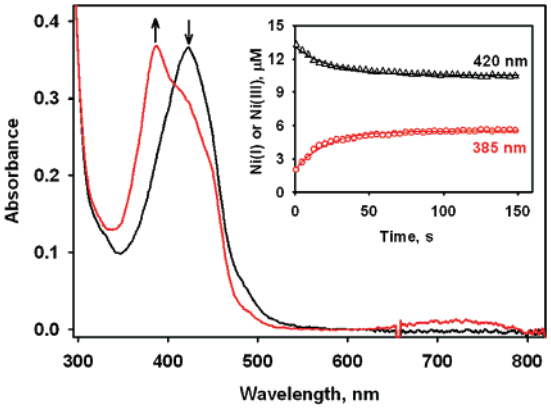
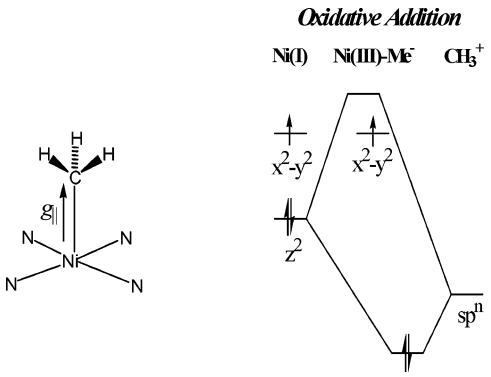


Figure 4. Conversion of MCR_{Me} (black) to MCR_{red1} (red). The inset shows decay of MCR_{Me} (Δ, 420 nm, k(decay) = 0.035(5) s^{-1}); formation of MCR_{red1} (○, 385 nm, k(form) = 0.044(5) s^{-1}). Conditions: MCR_{Me} formed in situ (10 μM MCR_{red1} plus 100 μM MeI in 50 mM Tris, pH 7.6) was treated with 10 mM HSCoM at 25°.

Scheme 1



Two competing mechanisms can contribute to the small transfer of spin to the axial ligand of a $d_{x^2-y^2}$ odd-electron metal ion: the low-symmetry environment of the Ni ion in F_{430} can introduce a small degree of mixing between $d_{x^2-y^2}$ and d_{z^2} and a resulting positive spin density (ρ_n) in the sp^n orbital on C; the $d_{x^2-y^2}$ spin can polarize the Ni−C bond, introducing negative spin density on C. The resultant ρ_n yields a spin density in the 2s orbital of carbon, $\rho_s = \rho_n/(n+1)$ and an isotropic coupling, $a(^{13}C) = \rho_s a_0(^{13}C)$, where $a_0(^{13}C) \approx 3770$ MHz is the coupling for a single 2s electron;[18] experiment then yields, $|\rho_s| \approx 0.01$.

Considering the ^{2}H ENDOR data, the fact that the isotropic interaction is small is consistent with the minimal transfer of spin to CH_3 revealed by the ^{13}C ENDOR results (small ρ_n), and suggests that the dipolar hyperfine interaction with ^{2}H is dominated by the through-space interaction with the Ni spin. In fact, the ^{13}C and ^{2}H hyperfine interactions can be self-consistently described[24] by the bonding in a Ni(III)−CH_3 fragment (Scheme 1) if we take a Ni−C bond length of 1.9−2.0 Å, C−H bond length of 1.0 Å, hybridization at C of $n \approx 2-3$, and $\rho_n \approx +0.04$. This interpretation can be examined with DFT computations.

If MCR_{Me} is a catalytic intermediate in methanogenesis and anaerobic methane oxidation, it should react with HSCoM to make methyl−SCoM and should undergo proton and electron transfers to form methane. For these reactions to be catalytic, MCR_{red1} must be regenerated during this process. When the CH_3−Ni(III) species was reacted with HSCoM, MCR_{red1} was regenerated with a rate constant of 0.044 s^{-1} (Figure 4) forming methyl−SCoM, as demonstrated by MS. Furthermore, addition of CoBSH to MCR_{Me} led to the formation of the CoB−SS−CoM heterodisulfide product, on the basis of MS coupled with MS−MS, and methane (Figure

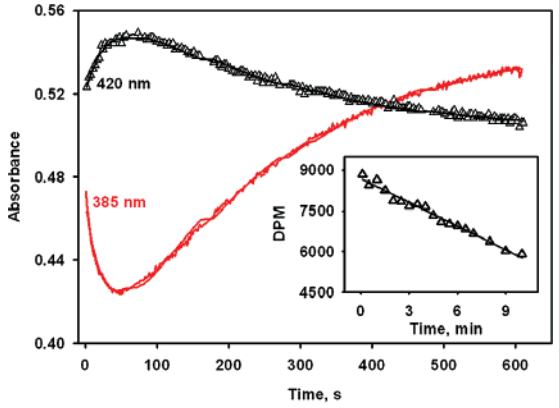


Figure 5. Formation of MCR_{Me} from MCR_{red1} and regeneration of MCR_{red1} by reaction of MCR_{Me} with Ti(III) citrate. MCR_{red1} (10 μM) was reacted with 50 μM CH_3I in 50 mM Tris, pH 7.6 in the presence of 0.1 mM Ti(III) citrate. Timecourses: MCR_{red1}, red trace, 385 nm; MCR_{Me}, 420 nm, open triangles. Fit to a three-component sequential reaction gave absorbance changes at 385 nm, $k_1 = 0.038(2)$ s^{-1}, $k_2 = 0.0047(2)$ s^{-1}; absorbance changes at 420 nm $k_1 = 0.033(4)$ s^{-1}, $k_2 = 0.0045(3)$ s^{-1}. The inset shows CH_4 formation from MCR_{Me} with Ti(III) citrate. MCR_{red1} (50 μM) was reacted with 1 mM $^{14}CH_3I$ in 50 mM Tris, pH 7.6 with 0.1 mM Ti(III) citrate; 10 μL aliquots of reaction mixture were quenched in THF at indicated times. Data was fit to a linear equation; specific activity was 0.0048 U mg^{-1} MCR_{red1} with $k = 0.044$ s^{-1}.

S3). These are the products of the reaction with the native substrates. Methane formation was shown by a steady-state experiment in which $^{14}CH_3$−Ni(III) (generated from $^{14}CH_3I$) was reacted with HSCoM to build up methyl−SCoM, at which point no methane was formed; then, upon incubation with CoBSH, methane was formed with a specific activity of 0.046 U mg^{-1} MCR_{red1} (Figure S4). When corrected to saturating methyl−SCoM concentrations, the rate constant would be 1.1 s^{-1}, similar to the typical rate of methane formation (4.5 s^{-1} at 20 °C).[6] Thus, the methyl−Ni intermediate can be converted to methyl−SCoM, regenerating active MCR_{red1} and, in the presence of CoBSH, can generate methane.

The low-potential reductant Ti(III) citrate is known to reduce MCR_{ox1} in vitro to active MCR_{red1}.[3,4] Figure 5 shows that MCR_{Me} also can undergo reduction by Ti(III) citrate to completely regenerate active MCR_{red1}, producing methane with a specific activity of 0.046 U mg^{-1} MCR_{red1} (k_{cat} of 0.011 s^{-1}) (inset, Figure 5). In this reaction, MCR_{Me} is generated in situ in the first phase of the reaction and then converted by Ti(III)−citrate to MCR_{red1}. Thus, the CH_3−Ni(III) species can undergo conversion to methane and regenerate MCR_{red1} in the presence of a low potential reductant (and a proton from solvent).

Support for the intermediacy of a methyl−Ni(III) intermediate during methanogenesis previously relied primarily on studies of inorganic models. Methane formation from the reaction of Ni(I)-derivatives of F_{430} and activated methyl donors like methylsulfonium ions has been observed[25] and a methyl−Ni(II) form of the pentamethyl ester of F_{430} has been characterized by NMR methods.[26] Furthermore, reaction of Ni(I)−octaethylisobacteriochlorin (a structural cousin of F_{430}) with alkyl halides generates alkyl-Ni(III) species that undergo reduction to the alkyl-Ni(II), subsequent protonolysis yielding the alkane.[27] The work described here is consistent with the intermediacy of the methyl−Ni species in methane formation, while the ENDOR measurements have characterized the methyl−Ni species long postulated to be the central catalytic intermediate of MCR.

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Supporting Information Available: Four figures showing EPR and ENDOR spectra, mass spectra, and a methane assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations: methyl-coenzyme M (methyl−SCoM), 2-(methylthio)-ethanesulfonic acid; coenzyme B (CoBSH), *N*-7-mercaptoheptanoyl-threonine phosphate; ENDOR, electron nuclear double resonance; mass spectrometry, MS.
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