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Methyl-Coenzyme M Reductase from Methanogenic Archaea: Isotope Effects on Label Exchange and Ethane Formation with the Homologous Substrate Ethyl-Coenzyme M

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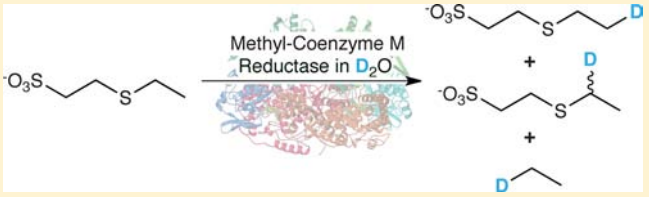
Supporting Information

ABSTRACT: Ethyl-coenzyme M (CH_3CH_2-S-CH_2CH_2-SO_3^-, Et-S-CoM) serves as a homologous substrate for the enzyme methyl-coenzyme M reductase (MCR) resulting in the product ethane instead of methane. The catalytic reaction proceeds via an intermediate that already contains all six C−H bonds of the product. Because product release occurs after a second, rate-limiting step, many cycles of intermediate formation and reconversion to substrate occur before a substantial amount of ethane is released. In deuterated buffer, the intermediate becomes labeled, and C−H activation in the back reaction rapidly leads to labeled Et-S-CoM, which enables intermediate formation to be detected. Here, we present a comprehensive analysis of this pre-equilibrium. ^{2}H- and ^{13}C-labeled isotopologues of Et-S-CoM were used as the substrates, and the time course of each isotopologue was followed by NMR spectroscopy. A kinetic simulation including kinetic isotope effects allowed determination of the primary and α- and β-secondary isotope effects for intermediate formation and for the C−H/C−D bond activation in the ethane-containing intermediate. The values obtained are in accordance with those found for the native substrate Me-S-CoM (see preceding publication, Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, DOI: 10.1021/ja406485z) and thus imply the same catalytic mechanism for both substrates. The experiment by Floss and co-workers, demonstrating a net inversion of configuration to chiral ethane with CH_3CDT-S-CoM as the substrate, is compatible with the observed rapid isotope exchange if the isotope effects measured here are taken into account.



INTRODUCTION

Methyl-coenzyme M reductase (MCR) catalyzes the last step of methanogenesis in all methanogenic archaea.[1] The reverse reaction is believed to be the first step in the process of anaerobic oxidation of methane with sulfate as the terminal electron acceptor.[2,3] MCR contains two molecules of the nickel cofactor F430 as prosthetic groups, which must be in their Ni(I) oxidation state for the enzyme to be active (Figure 1).

MCR converts methyl-coenzyme M (CH_3-S-CH_2CH_2-SO_3^-, Me-S-CoM) and the thiol coenzyme (CoB-SH) to methane and the corresponding disulfide, respectively. The mechanism of this reaction is unknown and lacks precedent in nonenzymic chemistry (see the preceding publication for a more comprehensive introduction, Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, 10.1021/ja406485z). The enzyme is highly specific for methyl-coenzyme M. The only *S*-alkyl-homologue that is known to be a substrate is ethyl-coenzyme M, which yields ethane as the product (Scheme 1).[4,5]

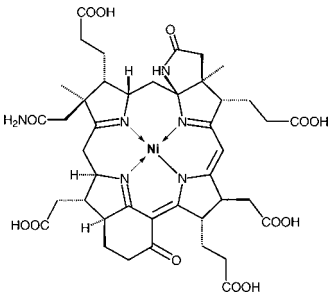


Figure 1. Coenzyme F430, the prosthetic group of MCR.

Intermediates in the Catalytic Cycle. The catalytic reaction of MCR involves at least one intermediate for both the native substrate methyl-coenzyme M and ethyl-coenzyme M.[6] The intermediate formed from ethyl-coenzyme M reacts about 100 times faster back to the substrate, than forward to free ethane. From label exchange experiments in deuterated medium it could be concluded that all six C−H bonds of ethane must already be present in the intermediate and that ethane is released only after a subsequent rate-limiting step. The isotope exchange pattern with a mono-^{13}C-labeled ethyl group in the substrate is depicted in Scheme 2.

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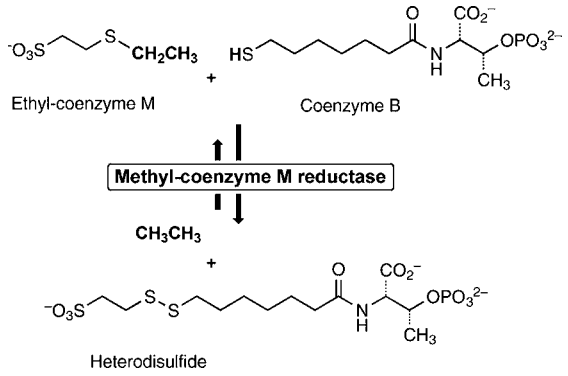
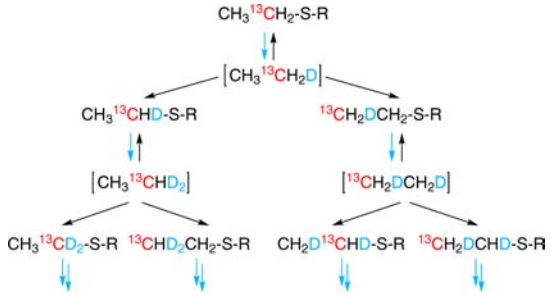
Scheme 1. Catalytic Reaction of MCR with the Homologous Substrate Ethyl-Coenzyme M Yielding the Product Ethane Instead of Methane



Scheme 2. Reaction of $CH_3{}^{13}CH_2$-S-CoM with CoB-SD and Enzyme in D_2O Buffer, Neglecting Ethane Formation[a]



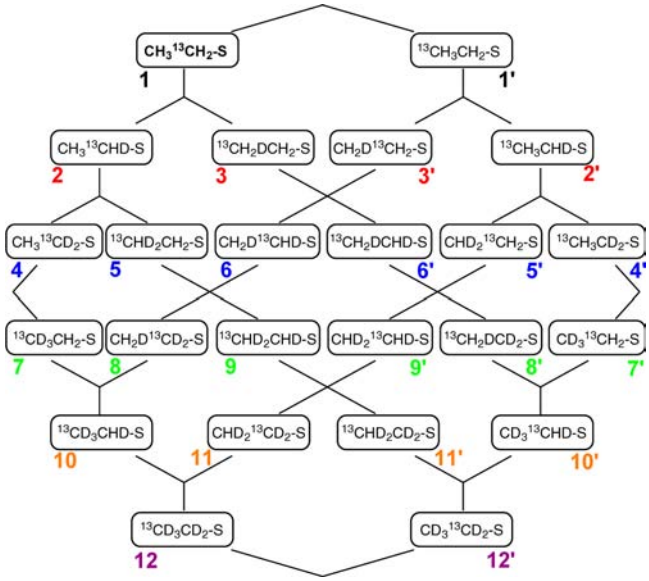
[a]The formation of the intermediates (depicted in brackets) is dependent on the solvent. In D_2O buffer, a deuterium atom is introduced into the intermediate (blue arrows), whereas in H_2O buffer a hydrogen atom would be introduced. The re-formation of substrates (black arrows) is independent of the H/D content of the solvent but is affected by primary and secondary isotope effects involving the C−H/C−D bonds in the intermediate.

With a mono-^{13}C-labeled ethyl group, 24 different isotopologues are possible (without counting enantiomers), which leads to the exchange sequence shown in Scheme 3.

Experiment to Deduce the Stereochemical Course of the Reaction to Chiral Ethane. Ahn and Floss incubated the chiral substrate CH_3CDT-S-CoM with a cell-free extract from *Methanosarcina barkeri* in order to obtain chiral ethane (CH_3CHDT), which was converted to acetate and analyzed for its configuration.[7] A net inversion of configuration was found (41% ee for the *S* and 51% ee for the *R* substrate). The finding of inversion of configuration is mechanistically significant, since it rules out a rotatable free ethyl radical or ethyl cation as an intermediate. At first sight, our discovery of rapid carbon scrambling and H/D exchange in the substrate ethyl-coenzyme M appears to be incompatible with the results obtained by Ahn and Floss. However, the competition between wash-out of deuterium (or tritium) and the formation of ethane depends on both conversion and potential isotope effects.

Here, we present the results of our study of all kinetic isotope effects (KIEs) involved in C−H bond formation and activation for the substrate ethyl-coenzyme M, which were extracted from kinetic simulations fitted to the observed time course for the label exchange in ethyl-coenzyme M. The determined KIEs will be compared with those obtained with the native substrate methyl-coenzyme M. In addition, ethyl-coenzyme M offered

Scheme 3. Isotope exchange sequence for ^{13}C-labeled ethyl-coenzyme M (isotopologues in Scheme 2 are: 1, 2, 3, 4, 5, 6, and 6′)[a]



[a]The intermediates are represented by crossings (or vertices) of the connecting lines; "CoM" parts are omitted for clarity. Each reaction in deuterated medium generates the intermediates on the next lower level; reaction in H_2O buffer generates intermediates on the next higher level. Shown is the numbering of isotopologues used in the Results section of this study. Isotopomers are labeled in the same color; ^{13}C-isotopomers are located at vertically symmetric positions. For experiments without ^{13}C-labeling, the identical nomenclature is used (species **1**−**12**, without an slanted prime).

the possibility to deduce β-secondary isotope effects. Our values for the KIEs were then used in a kinetic simulation of the Ahn and Floss experiment to judge the compatibility of their results with our measurements.

RESULTS

Label Exchange in $CH_3{}^{13}CH_2$-S-CoM as the substrate. Isotopologue **1**, ($CH_3{}^{13}CH_2$-S-CoM, 10 mM) was incubated with coenzyme B (2 mM) and active enzyme (0.94 μM) in D_2O buffer at 60 °C for 2, 4, 8, 16, and 32 min. The assay solutions were analyzed via $^{13}C\{^1H\}\{^2H\}$ NMR spectroscopy in order to quantify all isotopologues present, including those containing a fully deuterated ethyl group (Figure 2). A time course for each isotopologue is plotted in Figure 3.

Label Exchange in Deuterated Substrates. Deuterated ethyl-coenzyme M was incubated in H_2O buffer to study the wash out of deuterium. The isotopologues CD_3CD_2-S-CoM (**12**) and CH_3CD_2-S-CoM (**4**) were used and two different enzyme concentrations investigated. This set of isotope exchange experiments enabled extraction of the isotope effects for intermediate formation and for ethane activation in the intermediate. All relevant experiments are summarized in Table 1.

Experiments with the Isotopologue CH_3CD_2-S-CoM As the Substrate. The experiments with the isotopologue CH_3CD_2-S-CoM (**4**) as the substrate in H_2O buffer (experiments no 4 and 5) are discussed in more detail here, since incubation with MCR in H_2O buffer yields a mixture of only 5 different species (**1**−**5**, see Scheme 4) that can all be quantified accurately by ^{1}H NMR spectroscopy (Figure 4).

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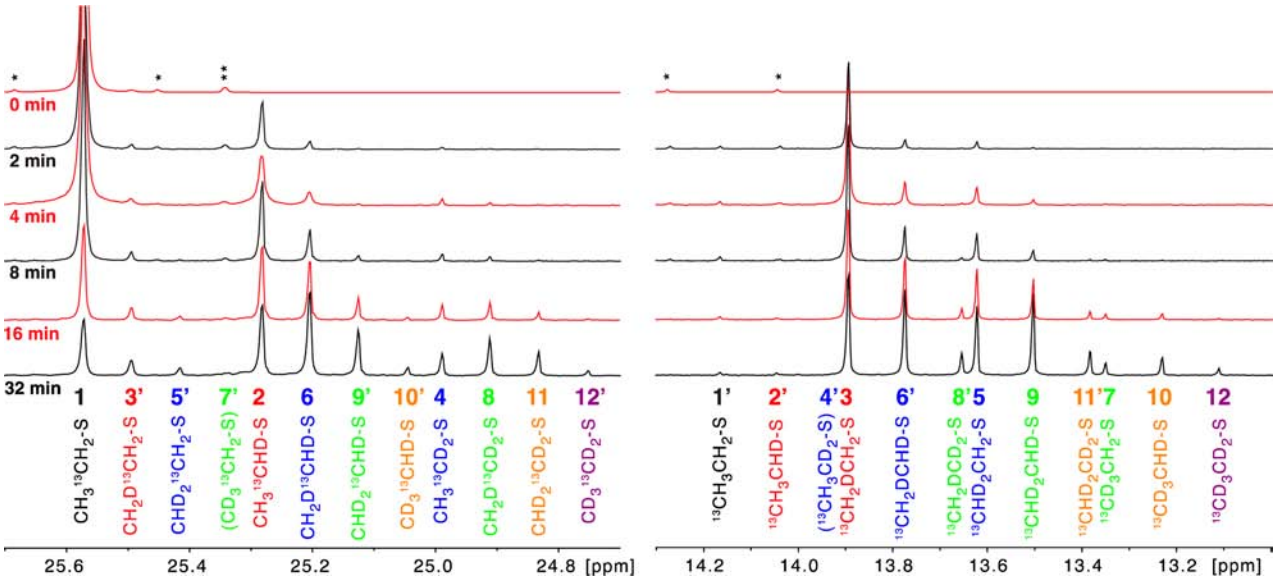

Figure 2. Expansion of the 150 MHz $^{13}C\{^1H\}\{^2H\}$ NMR spectra (simultaneous proton and deuterium broad-band decoupling) of the assay solutions before ("0 min") and after incubation with active enzyme at 60 °C for the time indicated. The numbering of isotopologues corresponds to that in Scheme 3. Lines marked with * are due to natural abundance of ^{13}C at the "unlabeled" position within the ethyl group. The signal marked with ** is due to natural abundance of ^{13}C at carbon 2 (Et-S-$^{13}CH_2CH_2$-SO_3^-). The fraction of isotopologues **4**′ and **7**′ remained below the detection limit.

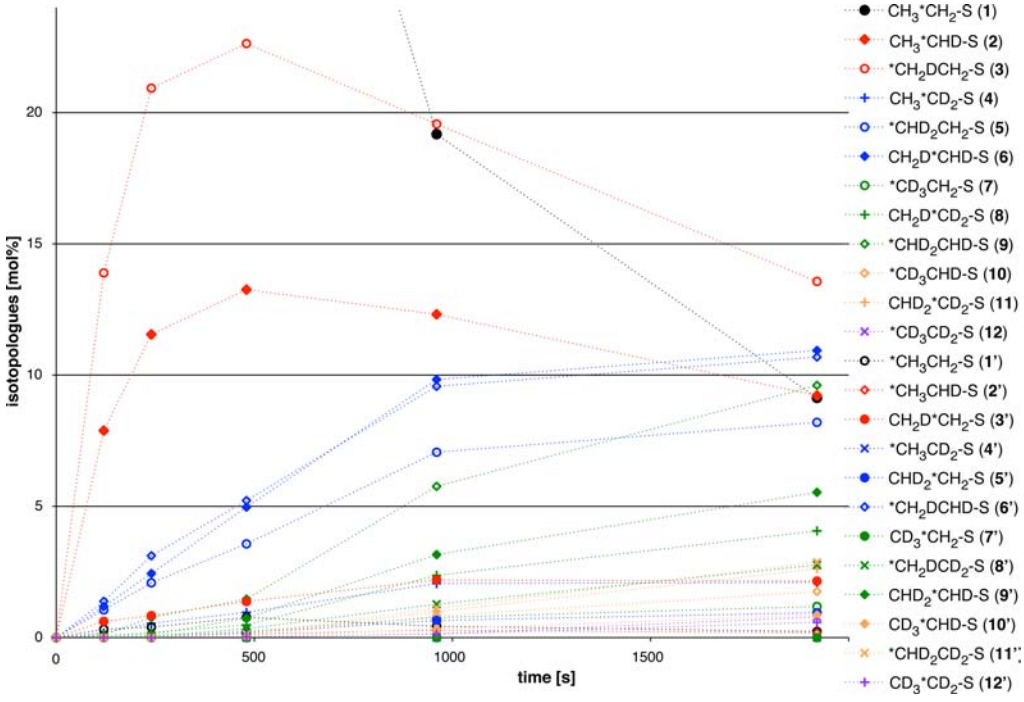

Figure 3. Time course of all 24 isotopologues during incubation of $CH_3{}^{13}CH_2$-S-CoM with MCR in deuterated medium. The molar fractions of the isotopologues were obtained via integration of the $^{13}C\{^1H\}\{^2H\}$ NMR spectra depicted in Figure 2 (inverse gated, 60 s relaxation delay). The isotopologues were normalized to 100% for each time point. The conversion to ethane at the last time point (1920 s) was about 5% as deduced from the amount of disulfides formed.

The $^1H\{^2H\}$ NMR spectrum (Figure 4) proves that only the expected isotopologues are present. The time course for each isotopologue (**1**−**5**) is plotted in Figure 5.

The isotope exchange pattern described in Scheme 4 matches the experiment. The initial substrate (isotopologue **4**) is first converted to isotopologues **5** and **2**. In a second step, isotopologues **3** and **1** appear. Visual inspection of Figure 5 already shows that substantial isotope effects must be involved in the C−H/C−D-activation in the intermediate $[CH_3CHD_2]$: **5** is formed ∼3 times more rapidly than **2**, while a ratio of 3:2 would be expected from statistics alone (number of H- and D-atoms, respectively).

Kinetic Modeling. In order to obtain the isotope effects involved in this exchange reaction, the sequence was simulated with the program COPASI,[8] (see Supporting Information [SI]

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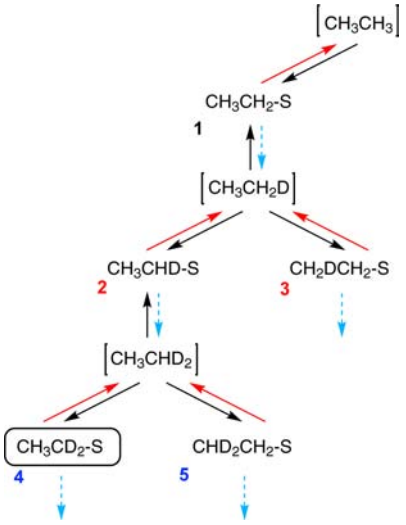
Table 1. Experiments Performed to Obtain a Time Course for Each Isotopologue

exp. no[a]	substrate[b]	solvent	enzyme [μM]
1a	$CH_3{}^{13}CH_2$-S-CoM (**1**)	D_2O	0.94
1b[c]	$CH_3{}^{13}CH_2$-S-CoM (**1**)	D_2O	0.94
2[c]	CH_3CH_2-S-CoM (**1**)	D_2O	0.94
3	CH_3CH_2-S-CoM (**1**)	D_2O	3.75
4	CH_3CD_2-S-CoM (**4**)	H_2O	0.94
5	CH_3CD_2-S-CoM (**4**)	H_2O	3.75
6	CD_3CD_2-S-CoM (**12**)	H_2O	0.94
7	CD_3CD_2-S-CoM (**12**)	H_2O	3.75

[a]Each experiment consisted of six samples that were incubated for 0, 2, 4, 8, 16, and 32 min, respectively at 60 °C. Exp. 1a was analyzed via $^{13}C\{^1H\}\{^2H\}$ NMR, exp. 1b−7 were analyzed via $^1H\{^2H\}$ NMR. [b]c = 10 mM. [c]Data reported earlier[6] and included in the kinetic simulation of isotope exchange reported here.

Scheme 4. Isotope Exchange Pattern for CH_3CD_2-S-CoM (4, box) in H_2O Buffer[a]



[a]Intermediates are represented in brackets. (Red pathways) Specific to H_2O buffer; the blue pathways would take place in D_2O buffer (dotted). Re-formation of substrates (black pathways) depends solely on the isotope effects involved.

S3.1 for a comprehensive description). The catalytic cycle was modeled in four reaction steps (Scheme 5).

For each step (Scheme 5), a rate constant was defined for both directions. Binding of the second substrate coenzyme B (step 2) defines whether hydrogen or deuterium will be introduced in the next step and ultimately depends on the isotopic composition of the buffer used (H_2O or D_2O).[9]

For the catalytic transformation (step 3, k_f), deuterium atoms in the CH_3- and -CH_2- positions of the ethyl group give rise to α and β secondary isotope effects on intermediate formation, which were both taken into account in the simulation. For the substrates labeled with ^{13}C, a $^{12}C/^{13}C$ KIE was included in the simulation.

In the reverse reaction (activation of a C−H/C−D bond of the ethane moiety in the intermediate; step 3, k_b), the same set of isotope effects was considered. In addition, the number of equivalent H or D leading to the same reformed substrate had to be taken into account as depicted in Scheme 6 for the first intermediate formed from isotopologue **1** in D_2O buffer.

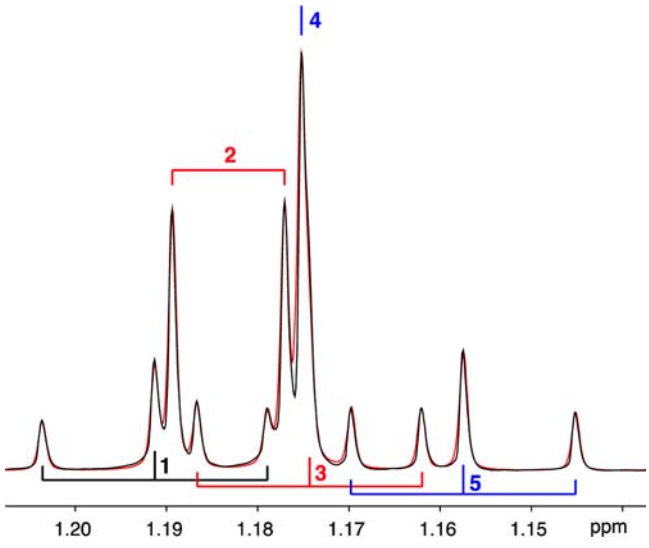


Figure 4. Expansion of the 600 MHz $^1H\{^2H\}$ NMR spectrum showing the methyl-group of Et-S-CoM after incubation with MCR for 32 min (exp. no 4). (Black line) Acquired spectrum. (Red line) Simulated spectrum. Assignments follow the numbering in Schemes 3 and 4

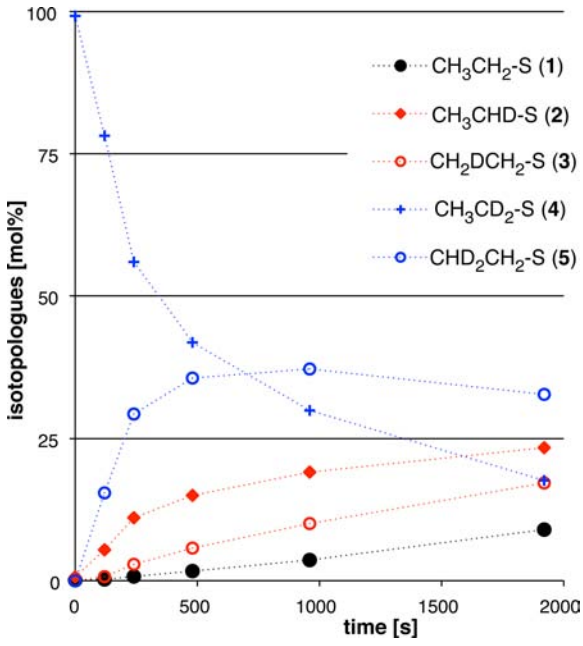


Figure 5. Time course of all isotopologues (**1**−**5**) obtained after incubation of CH_3CD_2-S-CoM in H_2O buffer containing coenzyme B and active enzyme.

The rate of release of ethane (step 4, k_{prod}) was adjusted to match the experiment and assumed to be independent of the isotopic constitution (see below for isotope effects on ethane release). To compare the experimental data more accurately with the simulation, all isotopologues that could be quantified (all for $^{13}C\{^1H\}\{^2H\}$ NMR analysis and only the H-containing ones for $^1H\{^2H\}$ NMR analysis) were normalized to 100% for both the experiment and for the simulation (see Experimental Procedures). The primary isotope effect (whether hydrogen or deuterium is introduced) corresponds to the ratio of enzyme activities in H_2O vs D_2O and was set to 1.0 (see below). Because of a gradual enzyme deactivation with increasing turnovers (later time points) and a delay in warming up to 60

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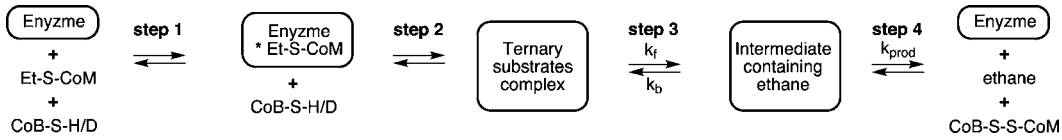
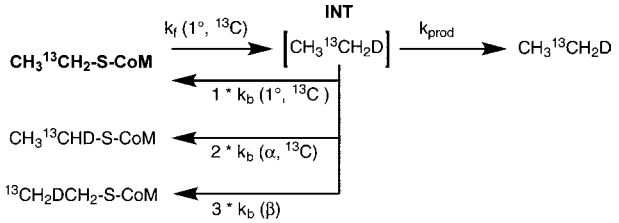
Scheme 5. Reaction Steps Simulated for the Isotope Exchange (steps 1−3) and for Ethane Formation (step 4)[a]



[a]**Step 1**: binding of the first substrate ethyl-coenzyme M. **Step 2**: binding of the second substrate coenzyme B. **Step 3**: catalytic step transforming the substrates ternary complex to the intermediate containing ethane (k_f, k_b). **Step 4**: This step is rate limiting and was simulated as a single elementary step with simultaneous release of both products (k_{prod}).

Scheme 6. Isotope Effects for C−H/C−D Bond Activation in the Intermediate That Were Taken into Account in the Kinetic Simulation, Exemplified for the Intermediate Formed from $CH_3{}^{13}CH_2$-S-CoM (1) as the Substrate in D_2O buffer[a]



[a]The rate constant for INT formation (k_f) and the rate constant for substrate re-formation (k_b) are modified through all isotope effects involved (specified in parentheses after the constant). 1° and ^{13}C signify primary KIEs, α and β signify secondary KIEs. The rate of C−H/C−D bond activation (k_b) is multiplied by the number of equivalent H/D atoms present.

°C (2-min time point), the experimental time scale had to be adjusted to obtain a constant reaction rate, enabling comparison with the simulation. Enzyme deactivation with increasing turnovers is commonly observed with MCR.[1] The relative time adjustment for each time point for all experiments is provided as S4.1 in the SI.

Extraction of Isotope Effects via Kinetic Modeling of the Exchange Reactions. Without isotope effects, the simulation could not be matched to the experiment, which signifies that IEs must be present and that step 4 is indeed rate-limiting. See S3.2 (in SI) for the simulation of experiment 5 from Table 1 without isotope effects (Figure S3.3 in SI) and for further discussion.

For each of the seven experiments, the combination of isotope effects yielding the best overall fit to the experiment was evaluated independently. For experiment 1, the analysis was carried out using $^{13}C\{^1H\}\{^2H\}$- and $^1H\{^2H\}$ NMR data independently. The best parameters extracted for each experiment are listed in Table 2.

Table 2. Individual Sets of Kinetic Isotope Effects Matching Each Experiment As Closely As Possible

KIE	1a[a]	1b[a]	2	3	4	5	6	7
$\alpha(k_f)$	1.2	1.25	1.3	1.2	1.2	1.2	1.2	1.2
$\beta(k_f)$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$1°(k_b)$	2.4	2.5	2.4	2.4	2.4	2.4	2.4	2.4
$\alpha(k_b)$	1.2	1.15	1.15	1.15	1.15	1.2	1.15	1.15
$\beta(k_b)$	1.0	1.0	1.0	1.0	1.05	1.1	1.0	1.0

[a]A ^{13}C KIE of 1.05 was assumed for k_f and for k_b, since a better fit to the experiment was obtained (see S3.5 in SI)

According to the kinetic model, all isotope effects have to be identical for all experiments. The values determined independently were averaged to give the values listed in Table 3, and a conservative confidence interval was estimated.

Table 3. Isotope Effects Averaged from All Simulations, Including the Estimated Confidence Intervals[a]

isotope effect	intermediate formation	C−H/C−D activation from intermediate
primary	1.0[b]	2.4 ± 0.2
α (per D)	1.22 ± 0.10	1.17 ± 0.10
β (per D)	1.0 ± 0.05	1.05 ± 0.05

[a]See Reliability of Isotope Effects below. [b]See below for determination.

Now, each experiment (1−7) was resimulated with the unified set of isotope effects (listed in Table 3) obtained through averaging over all experiments. A graph comparing each experiment with the unified simulation is provided in S3.3 in SI.

Reliability of the Isotope Effects Determined via Simulation. The value of each isotope effect was lowered and increased by the confidence interval estimated (Table 3) in order to determine its sensitivity to this parameter. Experiment 5 (reaction of CH_3CD_2-S-CoM (**4**) in H_2O at high enzyme concentration) was utilized for this purpose, because a double secondary isotope effect is involved (square of the intrinsic α-KIE). In addition, only five different species are formed in this experiment (see Scheme 4) that could all be determined accurately via ^{1}H NMR spectroscopy.

Variations of the α-secondary KIE for intermediate formation and the primary KIE for C−H/C−D bond activation in the intermediate are depicted in Figure 6. Variations of all other H/D KIEs are provided in S3.4 in SI, the variations of ^{13}C-KIEs are tested in experiment 1b (see S3.5 in SI for simulations with and without ^{13}C KIE).

The variations by the confidence intervals clearly result in a much poorer fit, which shows that the (rather large) confidence intervals estimated are reliable. Note that a large variation in the molar fraction of a specific isotopologue upon changing a particular isotope effect means that the simulation is sensitive to that isotope effect. Accordingly, the band between the thin line and the dotted line in Figure 6 can be regarded as the inverse of an error band.

Primary Isotope Effect on Intermediate Formation. The primary isotope effect on intermediate formation cannot be measured in a competitive experiment since it corresponds to the rate depression observed when the reaction is carried out in D_2O (experiments 1−3) instead of H_2O (experiments 4−7). However, it could be estimated from the ratio of the average simulated times vs experimental times, which correspond to the relative enzyme activities.

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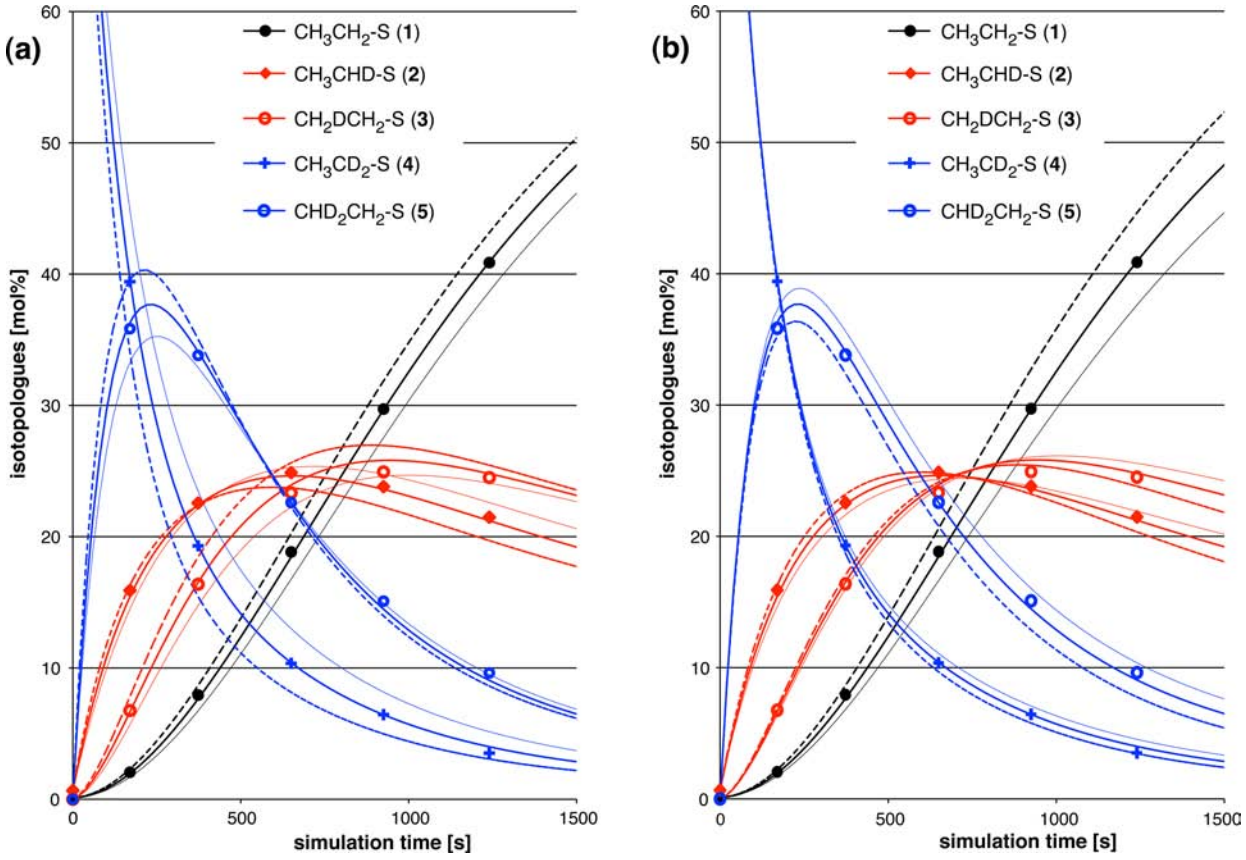

Figure 6. Overlay of measurements (points) and simulations (lines) for experiment 5, including the lower and upper limits for the isotope effects determined. (a) Variation of the α-secondary isotope effect on intermediate formation by the estimated confidence interval. (Solid line) Best value determined: KIE = 1.22. (Thin line) KIE = 1.32. (Dotted line) KIE = 1.12. (b) Variation of the primary isotope effect for the activation of the C−H/D bond in the ethane-intermediate by the estimated confidence interval. (Solid line) Best value determined: KIE = 2.4. (Thin line) KIE = 2.6. (Dotted line) KIE = 2.2). Corresponding variations of all other KIEs can be found in S3.4 (in SI). Note that the experimental times were adjusted in order to account for activity during warm-up and the enzyme deactivation generally observed with MCR (see S4.1 in SI for details).

The average activity for the assays in H_2O was found to be equal to that in the assays in D_2O, but the dispersion is large due to enzyme deactivation via oxidation of the Ni center in the enzyme (see S4.1 and S4.2 in SI). Because of the experimental scatter associated with enzyme activity, the primary kinetic isotope effect can only be delimited to the range 1.0 ± 0.3.

Testing for Enantioselectivity in the Formation of CH_3CHD-S-CoM. Substrate isotopologues with one D and one H in the methylene group are chiral. We investigated whether substrate re-formation to CH_3CHD-S-CoM (**2**) shows any enantioselectivity. Unlabeled ethyl-coenzyme M was incubated in D_2O buffer with CoB-SH and MCR to yield ∼20% conversion to monodeuterated isotopologues (CH_2DCH_2-S-CoM (**3**) and CH_3CHD-S-CoM (**2**)). After oxidation to sulfoxides, the ratio of the enantiomers for CH_3CHD-S-CoM was analyzed by ^2H NMR using (−)-dihydroquinine hemisulfate for chiral resolution (for details and spectra see S6 in SI) Additionally, experiments with CD_3CD_2-S-CoM in H_2O and with CD_3CH_2-S-CoM in D_2O, which both lead to the chiral isotopologue CD_3CHD-S-CoM (**10**), were carried out and investigated in the same way but via $^1H\{^2H\}$ NMR spectroscopy (data not shown). In all three experiments, the chiral isotopologues were formed as racemic mixtures. In the intermediate, the two hydrogens of the enzyme-bound $CH_3\mathbf{CH_2D}$ group are diastereotopic, in principle. The fact that C−H activation occurs at the same rate for H_{Re} and H_{Si} confirms that the intermediate contains a complete ethane moiety and that a possible steric isotope effect on the populations of the rotamers of the -CH_2D group is too small to be detectable (see S6.3 in SI for a detailed analysis).

Isotope Effect on the Formation of Free Ethane from the Intermediate. A potential isotope effect on step 4 was investigated by measuring the relative rate of ethane formation from the CH_3CH_3 intermediate and from the CD_3CHD_2 intermediate. To generate such a mixture of intermediates, a mixture of CH_3CH_2-S-CoM and CD_3CD_2-S-CoM was dissolved in H_2O buffer and incubated with CoB-SH and active enzyme. The IEs for step 3 influence the actual ratio of the two intermediates and have to be taken into account in order to calculate the IE for step 4 (Scheme 7).

A high concentration of ethyl-coenzyme M (200 mM) was used in this experiment in order to measure the initial rate and minimize relative isotope exchange. The ratio of undeuterated to pentadeuterated ethane found in the head space was analyzed via $^1H\{^2H\}$ NMR spectroscopy. The small amount of CHD_2CHD_2 and CD_3CH_2D formed was added to the amount of CD_3CHD_2 in the calculation of the isotope effect (Table 4).

In the absence of an IE for step 4 (k_{prod}), a ratio CH_3CH_3/CD_3CHD_2 of 1.49/2.89 = 0.515 would be expected for the overall reaction from an equimolar mixture of the two substrates to ethane due to the KIEs on step 3 (see S4.3 in SI for a detailed description).

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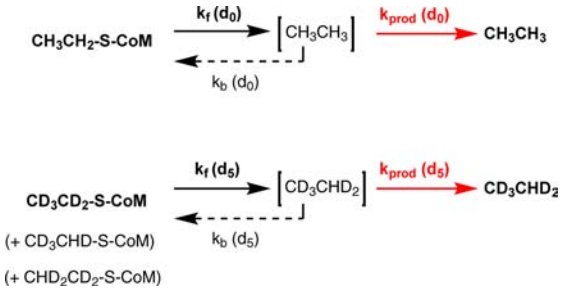
Scheme 7. Reaction Scheme for the Experiment To Determine the Isotope Effect on Ethane Formation with All Isotope Effects Involved[a]



[a]The ratio CH_3CH_3/CD_3CHD_2 (gas in the headspace) was measured. Red: the isotope effect on ethane formation from the intermediate, $k_{prod}(d_0)/k_{prod}(d_5)$, can be calculated, because all isotope effects for k_f and k_b are known from the simulation.

The isotope effect on k_{prod} (d_0 vs d_5) corresponds to the measured ratio (0.63) divided by the ratio expected without an IE on k_{prod} (0.515):

$$0.63 \pm 0.03/0.515 = 1.22 \pm 0.06.$$

Taking the errors for the KIEs on k_f and k_b into account, the errors accumulate, and we arrive at an IE for k_{prod} (d_0 vs d_5) = 1.2 ± 0.2.

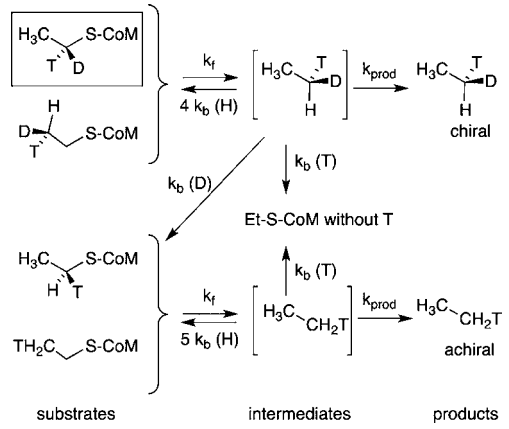
Simulation of the Experiment by Floss and Co-workers with the KIEs Determined in This Study. Using the KIEs obtained in this study we subsequently simulated the experiments determining the stereochemical course for ethane formation reported by Ahn and Floss.[7] A "chemical" kinetic model without the substrate-binding steps was simulated with the COPASI program (Scheme 8).

Both the forward and reverse-to-substrate reactions (k_f and k_b in Scheme 8) were assumed to occur under inversion of configuration. Initially, there are four species (2 mM total according to ref 7): (*R*)-CH_3CDT-S-CoM, (*S*)-CH_3CDT-S-CoM, (*R*)-CH_3CHD-S-CoM, and (*S*)-CH_3CHD-S-CoM. Their concentrations were set according to the % ee estimated for the substrate in ref 7 (75% max) and to the activities (1.6 and 2.8 μCi) reported. Most of the substrate is (*R*)-CH_3CHDSCoM and (*S*)-CH_3CHD-S-CoM (where H replaces T), again in a ratio corresponding to the % ee of 75%. The product CH_3CH_2T is counted but is achiral. In the *F*-value analysis of acetate, this species would be indistinguishable from racemic [1,1-D,T]-ethane (*F* value near 52%).

The % ee of formed ethane therefore corresponds to:

$$\%ee = \{|[(R)\text{-}CH_3CHDT] - [(S)\text{-}CH_3CHDT]|\} / \{[(R)\text{-}CH_3CHDT] + [(S)\text{-}CH_3CHDT] + [CH_3CH_2T]\}$$

Scheme 8. Kinetic System for the Simulation of the Loss of % ee Due to Isotope Exchange[a]



[a]Shown is the case starting with the (*R*)-substrate (box).

In the simulation, the commitment ($k_{prod}/4k_b$ in Scheme 8) was set to 0.00926, corresponding to our results obtained with the pure enzyme. The basic rate constant was set to give a total conversion of ~25% within 10 h (in Floss' experiments incubation with cell-free extract overnight gave conversions of 28% for the *R*-substrate and 10.3% for the *S*-substrate). Additional simulations were carried out, assuming only partial stereospecificity for the formation of the intermediate and re-formation of the substrate (k_f and k_b). The result of the simulations is shown in Figure 7.

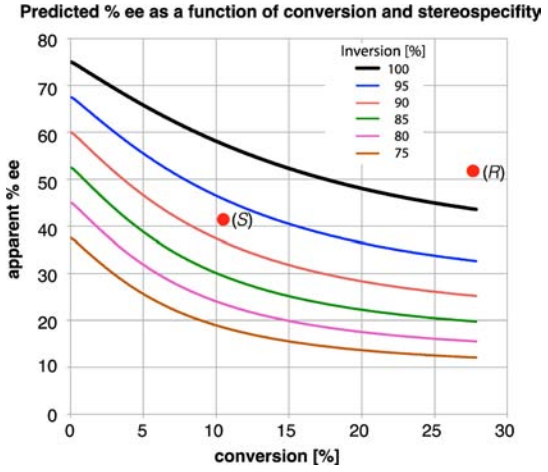


Figure 7. Simulation of the loss of % ee due to isotope exchange, taking into account the KIEs reported here and assuming complete inversion of configuration (solid black curve), and experimental values of ref 7 (red dots) for the two enantiomeric substrates (*R*)-CH_3CDT-S-CoM and (*S*)-CH_3CDT-S-CoM. Colored curves: simulations assuming only partial stereospecificity as indicated by inversion %.

Table 4. Isotope Effect on Ethane Formation Starting with a Mixture of Unlabeled Ethyl-Coenzyme M (1) and CD_3CD_2-S-CoM (12) As the Substrates[a]

time [min]	CH_3CH_3 [mol %]	CD_3CH_2D [mol %]	CHD_2CHD_2 [mol %]	CD_3CHD_2 [mol %]	sum d_4/d_5 [mol %]	ratio $d_0/(d_5 + d_4)$
2	38.1	1.1	1.1	59.7	61.9	0.615
4	38.8	1.6	1.6	57.9	61.2	0.635
8	39.0	2.5	2.5	55.9	61.0	0.641

[a]The ratio unlabeled/deuterated ethane measured at the three different incubation times averages to 0.63 ± 0.03.

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Whereas washout of deuterium leads to loss of chirality (CH_3CDT-S-CoM is replaced by CH_3CHT-S-CoM, which gives CH_3CH_2T as product), any partial nonstereospecificity in the formation of the intermediate and re-formation of substrate would lead to fast racemization of the substrate because, for each molecule going to product, 100 molecules are reacting back to substrate. In the simulations, this can be seen as a large initial drop of the % ee (colored curves in Figure 7).

Probing Propyl- and Allyl-S-CoM for Deuterium Incorporation. Propyl-coenzyme M and allyl-coenzyme M are not converted to propane or propene, respectively, but are known to be competitive inhibitors of MCR binding in the active site.[5] We investigated both inhibitors for deuterium incorporation analogously to the experiments with ethyl-coenzyme M, but no deuterium incorporation could be observed for either inhibitor, even at high enzyme concentrations.

DISCUSSION

Isotope Effects for Label Exchange in Ethyl-Coenzyme M. The KIEs for isotope exchange in ethyl-coenzyme M reported here correspond closely to those obtained for methane formation and methane activation, a strong indication that the reaction mechanism is the same for the two substrates. However, as reported earlier, different steps are rate-limiting if the substrate is methyl-coenzyme M (see preceding article) or ethyl-coenzyme M.[6] In order to compare the KIEs in the first catalytic step for the two substrates, one has to study methane formation in the case of methyl-coenzyme M but isotope exchange into the substrate for ethyl-coenzyme M.

The primary H/D KIE for the formation of the intermediate from substrate is small (1.0−1.3), while in the reverse direction, the primary H/D KIE of C−H activation from the intermediate is much larger (2.4 for both substrates). Substantial α-secondary KIEs of ca. 1.2/D were found in both directions for both substrates, indicating a change in hybridization from sp^3 toward sp^2 upon going to the transition state of step 3 in either direction.

The β-secondary KIEs (1.0 ± 0.05, 1.05 ± 0.05) found for ethyl-coenzyme M in the two directions of step 3 are not consistent with strong hyperconjugation in the transition state such as would be expected for an ethyl-cation-like TS, but congruent with the small β-secondary KIEs for homolytic cleavage to ethyl radical found in the literature (all <5%).[10]

In contrast to methyl-coenzyme M, the $^{12}C/^{13}C$-KIE could not be measured directly for ethyl-coenzyme M, but the simulations gave the best fit with k_{12}/k_{13} = 1.05, close to the value found for methyl-coenzyme M and consistent with breaking the carbon−sulfur bond in the first catalytic step.

Nature of the Ethane-Containing Intermediate. The fact that all six C−H bonds of ethane are already present and equivalent in the intermediate leaves only two structural possibilities: either noncoordinated ethane held in the active site by noncovalent forces or a (CH_3CH_3)Ni σ-complex. In the latter case, the dissociation energy of the σ-complex might contribute to the activation energy of the following, rate-limiting step (TS2). However, from the experimental IE on the overall reaction as determined in a competitive experiment with CH_3CH_2-S-CoM and CD_3CD_2-S-CoM we derive a KIE on the rate-limiting step that is close to unity (k_H/k_D = 1.2 ± 0.2). In contrast to *equilibrium* IEs,[11−16] no experimental data for *kinetic* isotope effects on the dissociation of alkane σ-complexes have been reported. In view of this lack of reference data, the small or absent KIE on TS2 does not allow us to distinguish between the two possible structures discussed here for the intermediate.

Radical Mechanisms. Since the IEs determined for ethyl-coenzyme M are very close to those found for methyl-coenzyme M, our assessment that the experimental IEs are qualitatively compatible with the radical-substitution mechanism of Siegbahn and Crabtree (Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, 10.1021/ja406485z) also holds for ethyl-coenzyme M. As with Me-S-CoM, a more quantitative comparison will require a DFT calculation for Et-S-CoM on the same level of theory and including vibrational analysis. It would be of particular interest whether such a calculation predicts equal activation energies for step 3 (TS1) for the two substrates as found by experiment (see below). No explanation for the observed difference in energy of TS2 relative to TS1 for the two substrates is derivable from the reported DFT calculation because the alkane was removed from the structural assembly for the calculation of the second step.[17] Hence, radical back-substitution of CoB-S● on the sulfur of Ni-coordinated coenzyme M thiolate and formation of the heterodisulfide and Ni(I)F430 is identical for both substrates.

The fact that no deuterium is incorporated into the competitive inhibitors allyl-coenzyme M and propyl-coenzyme is puzzling because their binding induces the same subtle changes in the EPR spectrum that are observed when Me-S-CoM is added to active MCR, thus confirming that these inhibitors bind in the active site like the natural substrate.[5] Since K_i^{app}(allyl-coenzyme M) = 0.2 mM is lower than K_M^{app}(Me-S-CoM) = 5 mM,[5] the active site of free MCR can accommodate the larger C_3 substrates in the binary substrate complex.

According to the calculated reaction profile of Chen et al., the late transition state of the first, endothermal step (TS1) contains an almost free alkyl radical.[17] Therefore, the energy of TS1 should directly reflect the stability of this radical (e.g., the BDE of the C−S bond) and the activation energy should decrease in the order methyl- > ethyl- ≫ allyl-coenzyme M. In order to try to explain the unreactivity of allyl-coenzyme M within the framework of the mechanism proposed by Siegbahn and co-workers one must assume that the larger *S*-alkyl substituents sterically hinder the binding of coenzyme B or the approach of its thiol group to the nascent alkyl radical.

Why Are the Steps after the Intermediate Rate Limiting for Ethyl-Coenzyme M but Not for Methyl-Coenzyme M? From the initial rate of methane formation from methyl-coenzyme M and the initial rate of ^{13}C scrambling in ethyl-coenzyme M the relative rates of the first catalytic step (step 3, TS1, k_f) for the two substrates can be estimated. Taking into account the different apparent K_M values (Me-CoM = 5 mM; Et-CoM = 20 mM)[5] the specific activities for formation of the intermediate from the substrate ternary complex are practically the same for the two substrates (Me-S-CoM: 20 U mg^{-1} and Et-S-CoM: 22 U mg^{-1}; both at saturation).[18] According to the kinetics of isotope exchange, the highest point in the reaction profile leading from the intermediate to products is 8.3 kJ mol^{-1} lower than the transition state of step 3 (TS1) for Me-S-CoM but 12.3 kJ mol^{-1} higher than TS1 for Et-S-CoM (see Scheme 4 in ref 6). Since both, C−S bond breaking and C−H bond formation occur in step 3 (TS1), why are the steps after the intermediate slower for ethyl-coenzyme M than for methyl-coenzyme M?

If the intermediate contains the free alkane, these subsequent steps comprise the formation of the heterodisulfide, which is chemically identical for both substrates, and the release of products. It is still unknown which one of the two products is released first. X-ray structures of inactive Ni(II) forms of the enzyme show a long narrow channel for entrance of the substrates that is completely plugged by bound coenzyme B ($MCR_{ox1\text{-}silent}$) or by the heterodisulfide product (MCR_{silent}).[19] This suggests that methane or ethane can only leave the active site after the heterodisulfide has been released. Rough estimates for the apparent K_M values for methane (>10 mM) and for the heterodisulfide (≤1 mM)[18] are in accord with this suggestion, because the K_M value of the first substrate is usually higher than that of the second substrate in ordered-bi bi mechanisms. In this picture of events, the reason why a step following intermediate formation becomes rate limiting with ethyl-coenzyme M as the substrate would have to be steric hindrance of either the formation or the release of the heterodisulfide by the slightly larger ethane molecule in a very tight active site.

Compatibility of Rapid Isotope Exchange with the Experiments Reported by Ahn and Floss. The substantial primary KIE of 2.4 for C−H bond cleavage (k_b) and the low conversion to free ethane reduce the effect of deuterium washout to the point where the predicted % ee is still 57 (expt with (*S*)-substrate: 41) at 10.3% and 43 (expt with (*R*)-substrate: 51) at 28% conversion. Taking into account the considerable experimental difficulties of the *F*-value determination reported by Ahn[20] and the fact that these authors studied cell free extracts containing MCR from *Methanosarcina barkeri* at 37 °C whereas our KIEs were obtained with MCR from *Methanothermobacter marburgensis* at 60 °C, the fast isotope exchange in ethyl-coenzyme M and the observation of net inversion of configuration by Ahn and Floss are not contradictory. However, it remains unexplained why the experimental %ee was higher for the run with higher conversion in ref 7.

CONCLUSION

Within experimental error, the kinetic isotope effects determined for C−H bond formation and C−H bond activation are identical for the native substrate Me-S-CoM and for its homologue Et-S-CoM, thus indicating that they react according to the same reaction mechanism. The large α-secondary KIEs and the fact that methyl- and ethyl-coenzyme M react at the same rate in the C−S bond breaking step speak against the earlier proposal of an S_N2 attack on carbon giving alkyl-Ni(III) species.[5,19,21] The value determined for the α-secondary IE (1.19 for Me-S-CoM and 1.22 for Et-S-CoM, per D) points to an $sp^3 \rightarrow sp^2$ hybridization change on going to the transition state, but the β-secondary IE near unity excludes a carbocation-like species.

Qualitatively, we judge the observed IEs to be compatible with the radical substitution mechanism proposed by Siegbahn and co-workers,[17] but a more quantitative comparison must await calculations of vibrational frequencies and IEs for both substrates. However, the fact that allyl-coenzyme M does not react or exchange isotopes at all, and that the relative energies of TS1 and TS2 are different for the two substrates are difficult to reconcile with this mechanism.

While the primary KIEs for C−H activation (k_H/k_D = 2.4−2.5) are similar to that observed by Bergmann for the cleavage in a putative alkane σ-complex,[22] no additional information substantiating or excluding such a complex as the intermediate could be obtained in our study.

The kinetic simulation of Floss' experiment showed that, when all isotope effects are taken into account, the reported[7] reduction of enantiomeric excess is fully attributable to isotope exchange in the substrate. This indicates that each elementary step in ethane formation is stereospecific with inversion of configuration as the overall result.

Our finding that ethyl- and methyl-coenzyme M show the same isotope effects, and therefore react according to the same catalytic mechanism, enhances the significance of all experiments done with ethyl-coenzyme M, among them the experiment by Floss and co-workers,[7] which cannot be carried out with the natural substrate methyl-coenzyme M.

EXPERIMENTAL PROCEDURES

Assay Conditions. The standard assays contained 10 mM of the desired isotopologue of ethyl-coenzyme M, 2 mM coenzyme B and an aliquot of MCR stock solution giving the amounts indicated. Coenzyme B is needed only catalytically for the isotope exchange. For the formation of ethane, the maximal fraction of conversion is 20%.

For each set of experiments, a stock solution (50 mM potassium phosphate buffer, pH = 7.6 in H_2O or D_2O, including the substrates) was prepared under ice cooling without enzyme. One aliquot was removed as a reference ("0 min value" or control). The remaining amount was supplemented with enzyme and distributed to different vials (1.60 mL into each). The volume of the liquid phase for all experiments was 1.60 mL. After being sealed with a rubber stopper, the vials were immediately incubated at 60 °C in a water bath shaker. The reaction was stopped after the desired reaction time by addition of 1 mL air with a syringe (immediate deactivation of MCR).

The remaining ethyl-coenzyme M was analyzed via NMR spectroscopy for its isotopic constitution. The fraction of conversion was determined by measuring the amount of disulfides formed. For the experiments measuring the product ethane, the gas was passed through $CDCl_3$ to absorb the ethane and measured via $^1H\{^2H\}$ NMR spectroscopy.

Isotope Exchange Experiments with Different Ethyl-Coenzyme M Isotopologues As Substrates. All seven experiments described in Table 1 were performed using the standard assay conditions described above with the same enzyme preparation. For experiments 1, 2, 4, and 6 the amount of enzyme was 1.5 nmol per assay (0.94 μM); for experiments 3, 5, and 7 an amount of 6.0 nmol (3.75 μM) was used (see Table 1).

For experiment 1, the analysis was carried out via integration of the $^{13}C\{^1H\}\{^2H\}$ NMR spectra (experiment 1a) and through fitting of the $^1H\{^2H\}$ NMR spectra (experiment 1b) independently. For experiment 1b, the following isotopologues were quantified: **1**−**6**, **8**, **9**, **11**, **6′**, **8′**, **9′**, and **11′**. For experiments 2, 3, 6, and 7: isotopologues **1**−**11** (all, except the fully deuterated isotopologue). For experiments 4 and 5: all isotopologues present (**1**−**5**).

Measurement of the Isotope Effect on Ethane Formation. The assays contained 100 mM CH_3CH_2-S-CoM, 100 mM CD_3CD_2-S-CoM, and 4 mM coenzyme B in H_2O buffer. After addition of 42 nmol enzyme (26.5 μM) per assay and closing the vial with the rubber stopper, the headspace was evacuated inside the anaerobic tent. The samples were incubated with the headspace under vacuum and stopped by addition of 1.0 mL of air. The headspace gas was removed with a gastight syringe and passed through $CDCl_3$ to analyze the formed ethane for its isotopic constitution. For details of the calculation of KIE on k_{prod} see S4.3 in the SI.

Probing Propyl- and Allyl-Coenzyme M for Isotope Exchange. Allyl-coenzyme M (10 mM) was tested for deuterium incorporation in a D_2O assay according to the standard assay conditions described above (enzyme: 15 nmol; 9.4 μM), incubation time: 32 min at 60 °C).

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Propyl-coenzyme M (10 mM) was tested using an assay containing a Ti(III)-citrate in D_2O (see preceding publication for details ([Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, 10.1021/ja406485z]). Enzyme: 1.28 nmol (0.8 μM), incubation time: 4 h at 60 °C).

^{1}H NMR analysis showed no deuterium incorporation for either inhibitor.

Kinetic Simulation with the Program COPASI. For the ratio of forward and backward reactions for substrate binding, the known apparent K_M values are taken into account. The rate constant for ethyl-coenzyme M binding was simulated fast (10^8 s^{-1}), and the catalytic step (step 3) was kept rate limiting. See SI (S3.1) for a detailed description of the entire model.

Purification of MCR. Protein purification was carried out following the procedure described in the preceding publication (Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, 10.1021/ja406485z). The concentration of the enzyme solution (~1 mL per purification) was ~0.7−1 mM, corresponding to 20−28% by weight. For each preparation, it was determined exactly by UV−vis spectroscopy. A typical UV−vis spectrum of an enzyme preparation is provided in the Supporting Information of the preceding paper (Scheller, S.; Goenrich, M.; Thauer, R. K.; Jaun, B. *J. Am. Chem. Soc.* **2013**, *135*, 10.1021/ja406485z).

NMR Measurements. ^{1}H NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer with an inverse detection probe. ^{2}H NMR spectra and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer with a cryodetection probe.

The assay solutions were centrifuged and 620 μL transferred into an NMR tube. After addition of 80 μL dioxane solution (0.025% v/v dioxane and 0.025% v/v dioxane-d_8 in D_2O) as an internal standard, the spectra were recorded at 278 K without spinning (30° excitation pulse, 5 s acquisition time).

Ethane was passed through $CDCl_3$ with a fine needle and measured with a 5 s relaxation delay (10 s repetition rate).

CW presaturation of the water signal for 2 s was employed for the assays in H_2O and 2 s relaxation delay for the samples in D_2O to ensure a uniform repetition rate of 7 s for all samples.

Assays with deuterium-containing isotopologues were acquired with and without broadband deuterium decoupling, denoted ^{1}H{^{2}H}.

^{2}H NMR measurements were carried out analogously to the ^{1}H NMR measurements, including D_2O presaturation where required. ^{2}H NMR measurements in undeuterated solvents (99% acetonitrile/1% H_2O) were carried out without lock.

For the ^{13}C NMR measurements with broadband ^{1}H and ^{2}H decoupling (inverse gated), a relaxation delay of 60 s was employed to allow reliable integration of the spectra.

The integration was carried out with the software BRUKER Topspin 2.1 relative to the internal standard dioxane (set to δ = 3.70 ppm for ^{1}H and for ^{2}H measurements, and set to δ = 67.0 ppm for ^{13}C NMR measurements).

The exact isotopic constitution in the ethyl group of ethyl-coenzyme M was determined by fitting of the spectra. The fraction of conversion was determined by comparing the amount of disulfides formed with the amount of remaining substrate, using the fittings of the CH_2 group at position 2: *R*-(*S*)-*S*-$\mathbf{CH_2}CH_2SO_3^-$ (for Et-S-CoM, CoB-S-S-CoM, and CoM-S-S-CoM). The ratio of different ethane isotopologues formed was determined by integration of the corresponding ^{1}H{^{2}H} NMR spectra.

Fitting of NMR Spectra. Fitting of NMR spectra was carried out with the software iNMR 4.1.7.

For each isotopologue, a spin system was defined for the ethyl group. The chemical shifts were adjusted for each α and β deuterium present ($\delta(CH_3)$ = 1.1743 ppm, α-shift = −17.0 ppb, β-shift = −8.13 ppb; $\delta(CH_2)$ = 2.5541 ppm, α-shift = −17.8 ppb, β-shift = −6.87 ppb). The proton coupling constant was $^3J_{H,H}$ = 7.4 Hz. The ^{13}C-coupling constants for the CH_3 group were: $^1J_{C,H}$ = 127.3 Hz, $^2J_{C,H}$ = 4.68 Hz; for the CH_2 group: $^1J_{C,H}$ = 139.5 Hz, $^2J_{C,H}$ =3.62 Hz. In addition, a ^{13}C-α-shift had to be applied and the line width was adjusted to the acquired spectra. (see S2.3 in the SI for a table of the NMR data of each isotopologue)

The simulated spectrum (sum of the lines calculated for all spin systems) was overlaid with the acquired spectra, and each parameter described above was fine-adjusted for each isotopologue.

Next, the population of each isotopologue was adjusted to match the measured spectrum (see S2.4 in the SI for overlays of all fitted and acquired spectra). The populations were normalized to mol % for comparison with the COPASI simulation and for plotting.

Tables of all normalized values of isotopologues can be found in S2.1 in the SI.

Synthesis of Substrates. Labeled and unlabeled substrates were synthesized as described previously.[6,23]

CH_3CD_2-S-CoM was prepared from CH_3CD_2I (Cambridge Isotope Laboratories). Scale: 1.72 g, 10.89 mmol; yield: 1.368 g, 7.04 mmol, 70%.

CD_3CD_2-S-CoM was prepared from CD_3CD_2I (Cambridge Isotope Laboratories). Scale: 1.90 g, 11.5 mmol; yield: 1.107 g, 5.6 mmol, 56%.

Propyl-S-CoM was prepared from propyl iodide (Aldrich). Scale: 4.42 g, 26.0 mmol; yield: 3.715 g, 18.0 mmol, 72%.

Allyl-S-CoM was prepared from allyl iodide (freshly distilled). Scale: 4.39 g, 26 mmol; yield: 3.03 g, 14.8 mmol, 59%.

CD_3CH_2-S-CoM was prepared from CD_3CH_2I (Cambridge Isotope Laboratories). Scale: 1.84 g, 10.6 mmol; yield: 1.077 g, 5.52 mmol, 55%.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures, details of experimental procedures, and acquired and measured NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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