Supporting Online Material for

Extending the Carbon Chain: Hydrocarbon Formation Catalyzed by Vanadium/Molybdenum Nitrogenases

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MATERIALS AND METHODS

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Natural abundance $^{12}$CO (99.5% purity) was purchased from Airgas (Lakewood, CA). All isotope-labeled compounds (≥ 98% isotopic purity) were purchased from Cambridge Isotopes (Andover, MA).

Protein Purification. *Azotobacter vinelandii* strains expressing wild-type VFe and MoFe proteins, and *vnfH*- and *nifH*-encoded Fe proteins were grown as described elsewhere (4). Published methods were used for the purification of these nitrogenase proteins (4).

Activity Determination. All nitrogenase activity assays were carried out in the presence of 100% CO at ambient temperature and pressure as previously described (7), except that the assays were scaled up by 130-fold from a standard reaction (4, 6, 7). In a 25 mL glass vial, each H$_2$O-based assay had a total volume of 13 mL and contained 25 mM Tris buffer (pH 8.0), 25 mM ATP, 52.5 mM MgCl$_2$, 300 mM phosphocreatine, 1.35 mg/mL phosphocreatine kinase, 20 mM dithionite, 200 mg VnfH or NifH and 20 mg VFe or MoFe protein. The D$_2$O-based assay had the same composition as the H$_2$O-based assay, except that all components were dissolved in 25 mM (D11)-Tris (i.e., (DOCD$_2$)$_3$CND$_2$) buffer and that all protein samples were exchanged into the same deuterated buffer. The pD of this buffer was adjusted to 8.0 with DCl and NaOD, determined by the previously established equation [pD = measured pH + 0.40 (24)], and further verified by pH indicator strips. Simultaneous determination of the hydrocarbon products was performed on an alumina F-1 column (Deerfield, IL). The products CH$_4$, C$_2$H$_4$, C$_2$H$_6$, C$_3$H$_6$,
C\textsubscript{3}H\textsubscript{8}, α-C\textsubscript{4}H\textsubscript{8}, and n-C\textsubscript{4}H\textsubscript{10}, as well as their deuterated counterparts, were quantified using a previously published method \((4, 25)\) as follows. First, varying amounts of purchased standards (0-1\%) were determined by a GC-FID equipped with an alumina F-1 column. Then, a linear standard curve \((R \geq 0.97)\) was constructed by plotting the known amounts of standards versus their respective, integrated areas. Finally, the samples were measured by the same GC-FID setup and the abundance of each given species in the samples was determined from the standard curve. The gas pressure of each injection was carefully normalized to obtain a consistent volume of analysis.

**GC-MS Analysis.** Samples were prepared as above, except that the reactions were terminated after 5 hr. GC-MS analysis was performed on an Agilent 6890 GC system coupled to a Waters GCT-Premier time-of-flight mass spectrometer. For each sample, 50 μL of gas was injected into a split/splitless injector, which was operated at 125\(^\circ\)C in split mode (30:1 split ratio). Gas separation was achieved with a PLOT-Q capillary column (0.320 mm ID x 30 m length), which was held at 40\(^\circ\)C for one min, increased to 120\(^\circ\)C at a rate of 5\(^\circ\)C/min, and held for an additional 3 min at 120\(^\circ\)C. Carrier He gas was passed through the column at 1.1 mL/min. The mass spectrometer was operated in electron impact ionization mode at 7000 resolution and calibrated over a range of 18 to 614 \textit{m/z} using reference H\textsubscript{2}O, N\textsubscript{2}, O\textsubscript{2}, Ar, and CO\textsubscript{2} in addition to ions from the mass reference compound tris(perfluoro-tributyl) amine. The calibrated mass axis was locked to the CF\textsubscript{3}\textsuperscript{+} ion at 68.995 \textit{m/z}.
**FIGURE S1**

**A** One-carbon product  
**B** Two-carbon products  
**C** Three-carbon products  
**D** Four-carbon products

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**Fig. S1.** Identities of hydrocarbons formed by V nitrogenase. GC-MS analyses of one (A)-, two (B)-, three (C)-, and four (D)-carbon products formed in the presence of 100% CO. The products were generated in the presence of H₂O (1 and 2) or D₂O (3 and 4) with ¹²CO (1 and 3) or ¹³CO (2 and 4) as the substrate. The mass-to-charge (m/z) ratios at which the products were traced are indicated in the figure. See Fig. S3 for the GC-based activity analyses of product formation and Fig. S4 for the representative GC traces of product distribution.
**Fig. S2.** Identities of hydrocarbons formed by Mo nitrogenase. GC-MS analyses of one (A)-, two (B)-, three (C)-, and four (D)-carbon products formed in the presence of 100% CO. The products were generated in the presence of H2O (1 and 2) or D2O (3 and 4) with 12CO (1 and 3) or 13CO (2 and 4) as the substrate. The mass-to-charge (m/z) ratios at which the products were traced are indicated in the figure. See Fig. S3 for the GC-based activity analyses of product formation and Fig. S4 for the representative GC traces of product distribution.
**FIGURE S3**

**A** V nitorgenase

**B** Mo nitorgenase

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Fig. S3. Time-dependent formation of hydrocarbons by V (A) and Mo (B)-nitrogenases. Formation of CH₄ (1), C₂H₂ (2), C₂H₆ (3), C₃H₆ (4), C₃H₈ (5), α-C₄H₈ (6) and n-C₄H₁₀ (7) in the presence of H₂O (●-) or D₂O (○-) over a time period of 120 min. Data are presented as mean ± SD (N = 5).
**Fig. S4.** Gas chromatography of hydrocarbon products formed by V (A, B)- and Mo (C, D)-nitrogenase-catalyzed reactions in H₂O (A, C) and D₂O (B, D). The CO-background was subtracted from all traces.
### TABLE S1

Table S1. Alkene/alkane ratios of V- and Mo-nitrogenases.

<table>
<thead>
<tr>
<th>Products</th>
<th>V nitrogenase</th>
<th>Mo nitrogenase</th>
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<tr>
<td></td>
<td>H₂O</td>
<td>D₂O</td>
<td>H₂O</td>
<td>D₂O</td>
<td></td>
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<tr>
<td>Methane</td>
<td>CH₄ or CD₄</td>
<td>--</td>
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<tr>
<td>Two-carbon products</td>
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<td></td>
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<tr>
<td>Ethylene</td>
<td>C₂H₄ or C₂D₄</td>
<td>31.78</td>
<td>22.46</td>
<td>2.00</td>
<td>6.92</td>
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<tr>
<td>Ethane</td>
<td>C₂H₆ or C₂D₆</td>
<td>0.08</td>
<td>0.38</td>
<td>0.50</td>
<td>0.89</td>
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<td>Three-carbon products</td>
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<tr>
<td>Propylene</td>
<td>C₃H₆ or C₃D₆</td>
<td>0.60</td>
<td>0.68</td>
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<td>0.92</td>
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<td>Four-carbon products</td>
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<td>α-Butylene</td>
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</table>

All ratios were calculated based on the data in Figure 2.
References and Notes


5. A small-scale nitrogenase reaction typically features 0.15 mg VFe or MoFe protein, the catalytic component of V or Mo nitrogenase. Such an assay condition was established empirically some 30 years ago (6) and has since been used as the conventional scale of in vitro nitrogenase assays in the field.


8. Based on the retention time on a gas chromatograph column, the low–molecular-weight hydrocarbon products formed by the Val¹⁰⁷⁰-substituted Mo nitrogenase variants have been assigned as methane, ethylene, ethane, propylene, and propane (9).


12. Materials and methods are available as supporting material on *Science* Online.

13. Given the detection threshold of CH₄ (0.0007 nmol per nmol protein per min) and the activity of V nitrogenase in CH₄ production (0.12 nmol per nmol protein per min), the Mo nitrogenase is at least 170-fold less active than its V counterpart in catalyzing the formation of CH₄ from CO.

14. Only 0.04% of electrons can be traced in the hydrocarbon products formed in the Mo nitrogenase–catalyzed reaction.

16. The effect of deuterium on nitrogenase-catalyzed CO reduction is probably multifaceted. Apart from the inverse kinetic isotope effects (i.e., $k_H/k_D < 1$, where $k_H$ and $k_D$ are the rate constants of H$_2$O- and D$_2$O-based reactions, respectively) of D$_2$O that favor the formation of deuterated products (18, 19), other solvent effects of D$_2$O—such as those affecting the protein conformation, the interaction between proteins, and the network of hydrogen bonds—have been observed (20–23).


