Biocatalysis

DOI: 10.1002/anie.201108916

ATP-Independent Formation of Hydrocarbons Catalyzed by Isolated Nitrogenase Cofactors**

Chi Chung Lee, Yilin Hu, and Markus W. Ribbe**

Nitrogenases are highly complex and uniquely versatile metalloenzymes that are capable of reducing a broad spectrum of substrates, such as N_2, CO, and CN^- ions, under ambient conditions.[1–4] The molybdenum- and vanadium-nitrogenases are two homologous members of this enzyme family, both of which utilize a specific reductase (Fe protein) to donate electrons to the cofactor site (FeMoco or FeVco) of a catalytic component (MoFe or VFe protein) during catalysis. The buried location of the cofactor poses a challenge for electron transfer in this process and renders it strictly dependent on the ATP-assisted formation of an electron-transport chain (within a complex between the reductase and the catalytic component) that extends all the way from the $[Fe_4S_4]$ cluster of the reductase, through the P-cluster, to the cofactor site of the catalytic component.[5] On the other hand, both FeMoco and FeVco can be extracted as intact entities into organic solvents,[6–8] which has spurred interest in seeking an ATP-independent reaction system in which electrons can be directly delivered to the isolated cofactors for reduction of a substrate. In particular, the recent discovery that nitrogenases can reduce CO to hydrocarbons[3,4] makes it attractive to explore the capacity of cofactors to directly catalyze the formation of hydrocarbons from CO, as well as CN^- ions, which are isoelectronic with CO.

Such a task can be accomplished by extracting the cofactors with *N*-methylformamide (NMF)[6–8] and combining them with a strong reductant, europium(II) diethylenetriaminepentaacetate [Eu^{II}–DTPA],[9] in an ATP-free buffer system. The isolated cofactors remain sufficiently stable in this buffer system, keeping more than 90% integrity within the first hour (Figure S1 in the Supporting Information) and thereby permitting the determination of the activity over this time period. Driven by [Eu^{II}–DTPA] ($E^{0\prime} = -1.14$ V at pH 8), both FeMoco and FeVco reduce CO to CH_4, C_2H_4, C_2H_6, C_3H_6, C_3H_8, 1-C_4H_8, *n*-C_4H_{10}, and 1-C_5H_{10}, under ambient conditions (Figure 1; see also Figure S2 in the Supporting Information). When CN^- ions are used as a substrate, the same set of products are generated together with NH_3 in both FeMoco- and FeVco-based reactions. In the reaction catalyzed by FeMoco, *n*-C_5H_{12}, 1-C_6H_{12}, *n*-C_6H_{14}, and *n*-C_7H_{16} are also detected as products (Figure 1; see also Figure S2 in the Supporting Information). The product profiles for the reduction of CN^- ions and CO are similar, which is consistent with a previously proposed, common C–C coupling pathway.[10] However, the rates of product formation in the reaction with CN^- ions are considerably higher than those in the reaction with CO, which likely results from a stabilizing effect of CN^- ion-binding on the isolated cofactors.[11] GC-MS analysis further confirms that CO and CN^- ions are the carbon sources for the hydrocarbons that are generated in these reactions, as all of the products display the expected mass shifts when ^{12}CO and $^{12}CN^-$ ions are replaced by ^{13}CO and $^{13}CN^-$, respectively, in the reaction (Figure 2).

There are interesting discrepancies in how the cofactors react with the two carbonaceous substrates in the solvent-extracted/[Eu^{II}–DTPA]-driven and protein-bound/ATP-driven states. Both isolated cofactors are less active than their protein-bound counterparts for the reduction of CO; however, the total amounts of hydrocarbons formed by isolated FeMoco and FeVco are 67.9% and 0.05%, respectively, of the totals produced by the protein-bound FeMoco and FeVco.[3,4,12] Such a disparate decrease in CO-reducing efficiency renders FeMoco, which is only 0.1% as active as FeVco within the protein, comparably active with FeVco in the isolated state (Figure 1). With regard to CN^- ions, the protein-bound cofactors normally reduce this substrate to CH_4 and NH_3.[1] This is not the case when CN^- ions are reduced by the isolated cofactors, as CH_4 is no longer the major carbonaceous product, and alkenes/alkanes of two to seven carbon atoms in length are also detected as products in this reaction (Figure 1).

The differences between the isolated and protein-bound cofactors in hydrocarbon formation highlight the significant impact of the protein environment on the reactivity of nitrogenase cofactors.[13] Nevertheless, the ability of isolated cofactors to catalyze the ATP-independent formation of long-chain, liquid-phase hydrocarbons suggests the possibility of developing electrocatalysts for the production of fuel under ambient conditions. Understanding how nitrogenases catalyze the formation of hydrocarbons is crucial for achieving this goal. These enzymes not only provide a prototype for such an electrocatalyst, but also serve as a biological blueprint for a synthetic matrix that immobilizes the catalyst and mimics the protein machinery for enhanced, ATP-independent electron transfer.

[*] C. C. Lee, Dr. Y. Hu, Prof. Dr. M. W. Ribbe
Molecular Biology and Biochemistry
University of California, Irvine
2236/2448 McGaugh Hall, Irvine, CA 92697-3900 (USA)
E-mail: yilinh@uci.edu
mribbe@uci.edu

[**] We thank Prof. Dr. D. C. Rees and Dr. Nathan Dalleska of Caltech (Pasadena) for help with the GC-MS analysis. This work was supported by Herman Frasch Foundation grant 617-HF07 (M.W.R.) and NIH grant GM-67626 (M.W.R.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201108916.

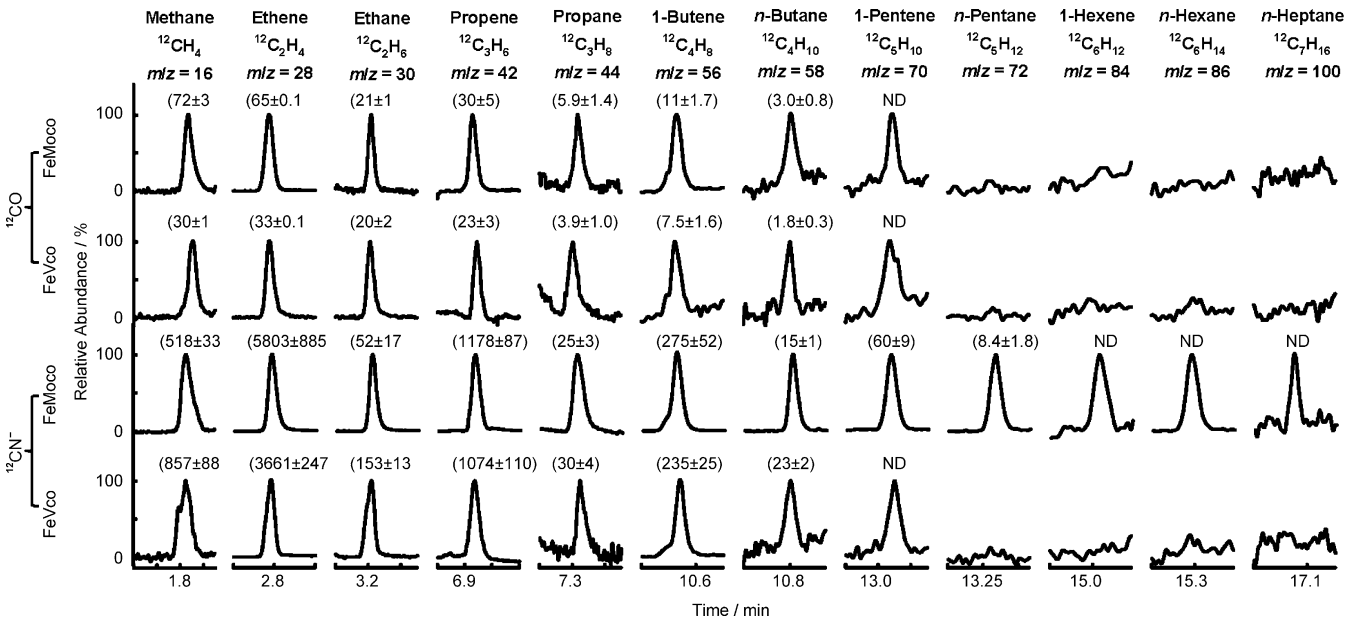

Figure 1. GC-MS analysis of hydrocarbons generated from the reduction of ^{12}CO and $^{12}CN^-$ ions by isolated cofactors. Specific activities of hydrocarbon formation are stated in nmol per μmol of cofactor per hour above the corresponding traces and presented as (mean ± standard deviation) ($N = 5$). ND = not determined by GC-based activity analysis. For time-dependent formation of these products, see Figure S2 in the Supporting Information. Other than hydrocarbons, NH_3 is formed at (18270 ± 129) and (15128 ± 107) nmol per μmol of cofactor per hour in the FeMoco- and FeVco-catalyzed reduction of CN^- ions, respectively. Relative to the amounts of nitrogen that appear in NH_3, the total amounts of carbon that appear in hydrocarbons are 95% and 85% in the FeMoco- and FeVco-catalyzed reduction of CN^- ions, respectively.

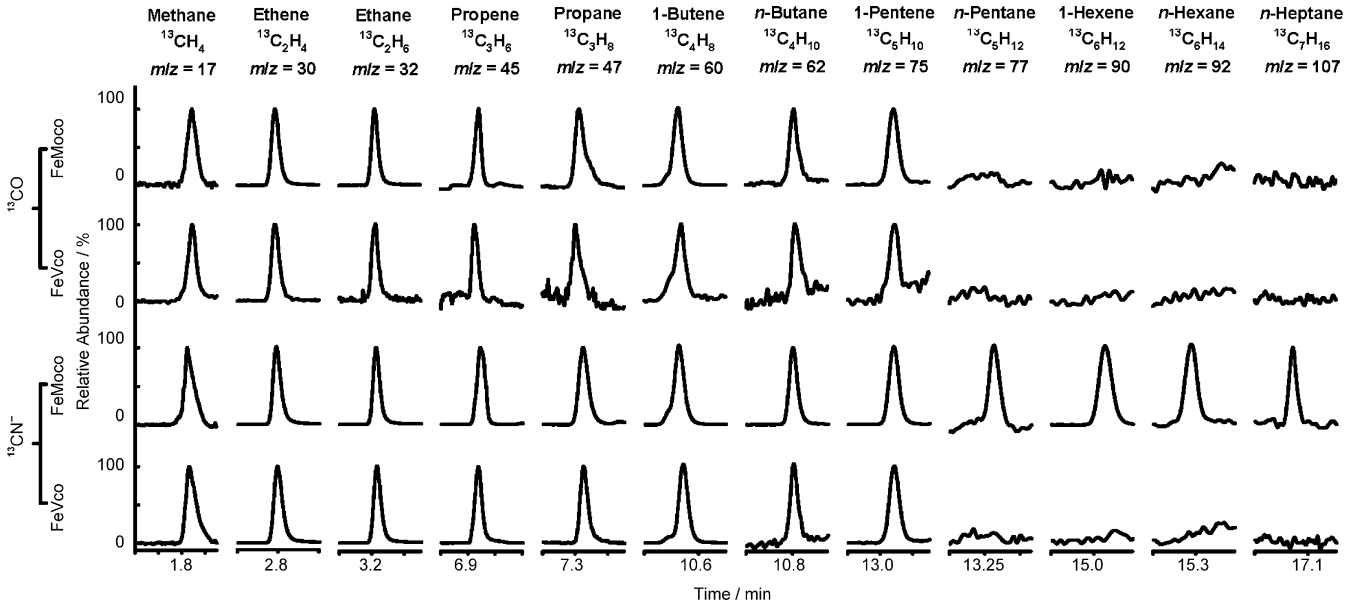

Figure 2. GC-MS analysis of hydrocarbons generated by isolated cofactors from the reduction of ^{13}CO and $^{13}CN^-$ ions.

Experimental Section

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 and cofactor extraction: *Azotobacter vinelandii* strains expressing His-tagged VFe and MoFe proteins were grown as described elsewhere.[14] Published methods were used for the purification of these nitrogenase proteins.[14] FeVco and FeMoco were extracted into NMF from VFe protein (1.5 g) and MoFe protein (1.5 g), respectively, by using a previously described method.[6]

Reduction of CN^- ions and CO by isolated cofactors: A stock solution of [Eu^{II}–DTPA] was prepared by dissolving equal molar amounts of europium(II) chloride and diethylenetriaminepentaacetic acid at a final concentration of 200 mM in 1M Tris buffer (pH 8.0). The reduction reaction with CN^- ions contained Tris (25 mM, pH 7.8), [Eu^{II}–DTPA] (5 mM),[9] isolated FeMoco or FeVco (0.4 mL), and

NaCN (100 mM) in a total volume of 25 mL. The reduction reaction with CO was of the same composition, except that CO (100%) was used instead of NaCN. Both reactions were run at room temperature and pressure for varying lengths of time. For GC-MS experiments with isotope labels, natural abundance $Na^{12}CN$ and ^{12}CO were replaced by $Na^{13}CN$ and ^{13}CO, respectively.

Activity analysis of the reduction of CN^- ions and CO: The formation of products CH_4, C_2H_4, C_2H_6, C_3H_6, C_3H_8, 1-C_4H_8, *n*-C_4H_{10}, 1-C_5H_{10}, *n*-C_5H_{12}, 1-C_6H_{12}, *n*-C_6H_{14}, and *n*-C_7H_{16} was determined by GC on an activated alumina column (Grace, Deerfield, IL), which was held at 40 °C for 2 min, heated to 200 °C at 10 °C min^{-1}, and held at 200 °C for another 2 min. The twelve hydrocarbon products were quantified as described previously[3,4] and their detection thresholds were, 1.1 (CH_4), 1.3(C_2H_4), 1.3 (C_2H_6), 1.5 (C_3H_6), 1.3 (C_3H_8), 1.4 (1-C_4H_8), 1.4 (*n*-C_4H_{10}), 3.1 (1-C_5H_{10}), 3.7 (*n*-C_5H_{12}), 6.1 (1-C_6H_{12}), 5.8 (*n*-C_6H_{14}) and 7.0 (*n*-C_7H_{16}) nmol per μmol of cofactor. NH_3 was determined by an HPLC fluorescence method.[15]

Determination of cofactor stability: The stability of isolated FeMoco or FeVco was determined by its ability to reconstitute the cofactor-deficient Δ*nifB* MoFe protein after incubation with the buffer system that was used for the [Eu^{II}–DTPA]-driven reduction reactions with CO and CN^- ions. Specifically, the isolated FeMoco or FeVco was incubated with the buffer system for varying lengths of time before Δ*nifB* MoFe protein was added to the mixture and the resulting reconstituted MoFe protein was assayed for activity.[7]

GC-MS: The hydrocarbon products were identified by GC-MS on a Hewlett–Packard 5890 GC and 5972 MSD. The identities of CH_4, C_2H_4, C_2H_6, C_3H_6, C_3H_8, 1-C_4H_8, *n*-C_4H_{10}, 1-C_5H_{10}, *n*-C_5H_{12}, 1-C_6H_{12}, *n*-C_6H_{14}, and *n*-C_7H_{16} were confirmed by comparing the masses and retention times with those of the Scott standard *n*-alkane and 1-alkene gas mixture (Plumsteadville, PA). A total of 50 μL of gas was injected into a split/splitless injector operated at 125 °C in splitless mode. A liner (1 mm internal diameter (ID)) was used to optimize the sensitivity. Gas separation was achieved on a Restek PLOT-QS capillary column (Bellafonte, PA; 0.320 mm ID × 30 m length), which was held at 40 °C for 1 min, heated to 220 °C at 10 °C min^{-1}, and held at 220 °C for another 3 min. The He carrier gas was passed through the column at 1.0 mL min^{-1}. The mass spectrometer was operated in electron impact (EI) ionization and selected ion monitoring (SIM) mode.

Received: December 18, 2011
Revised: January 3, 2012
Published online: January 17, 2012

Keywords: carbon monoxide · cofactors · enzymes · hydrocarbons · nitrogenases

[1] B. K. Burgess, D. J. Lowe, *Chem. Rev.* **1996**, *96*, 2983.
[2] R. R. Eady, *Chem. Rev.* **1996**, *96*, 3013.
[3] C. C. Lee, Y. Hu, M. W. Ribbe, *Science* **2010**, *329*, 642.
[4] Y. Hu, C. C. Lee, M. W. Ribbe, *Science* **2011**, *333*, 753.
[5] H. Schindelin, C. Kisker, J. L. Schlessman, J. B. Howard, D. C. Rees, *Nature* **1997**, *387*, 370.
[6] A. W. Fay, M. A. Blank, C. C. Lee, Y. Hu, K. O. Hodgson, B. Hedman, M. W. Ribbe, *J. Am. Chem. Soc.* **2010**, *132*, 12612.
[7] A. W. Fay, C. C. Lee, J. A. Wiig, Y. Hu, M. W. Ribbe, *Methods Mol. Biol.* **2011**, *766*, 239.
[8] V. K. Shah, W. J. Brill, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3249.
[9] K. A. Vincent, G. J. Tilley, N. C. Quammie, I. Streeter, B. K. Burgess, M. R. Cheesman, F. A. Armstrong, *Chem. Commun.* **2003**, 2590.
[10] E. M. Carnahan, J. D. Protasiewicz, S. J. Lippard, *Acc. Chem. Res.* **1993**, *26*, 90.
[11] C. J. Pickett, K. A. Vincent, S. K. Ibrahim, C. A. Gormal, B. E. Smith, S. A. Fairhurst, S. P. Best, *Chem. Eur. J.* **2004**, *10*, 4770.
[12] These numbers were derived from comparisons between the activities of protein-bound and solvent-isolated cofactors and are given in nmol of total carbon in hydrocarbon products per μmol of cofactor per hour.
[13] Although comparable in activity in the current study, the isolated FeVco, similar to its protein-bound counterpart, may be much more active than the isolated FeMoco for the reduction of CO. The [Eu^{II}–DTPA] system used in this work may have a limited capacity to deliver electrons to the two cofactors, which renders them similarly inefficient in the isolated state.
[14] C. C. Lee, Y. Hu, M. W. Ribbe, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9209.
[15] J. L. Corbin, *Appl. Environ. Microbiol.* **1984**, *47*, 1027.

