VIP C–C Formation

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Tracing the Hydrogen Source of Hydrocarbons Formed by Vanadium Nitrogenase**

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Nitrogenase catalyzes the biological reduction of dinitrogen (N_2) to ammonia (NH_3), a key step in the global nitrogen cycle.[1] The vanadium (V) and molybdenum (Mo) nitrogenases, two closely related members of this metalloenzyme family, share a good degree of homology both in primary sequence and in cluster topology. Both nitrogenases are binary systems comprising an adenosine triphosphate (ATP)-dependent reductase (*vnfH*- or *nifH*-encoded Fe protein) and a catalytic component (*vnfDGK*-encoded VFe protein or *nifDK*-encoded MoFe protein).[2] In addition, both systems presumably follow the same mode of action during substrate turnover, that is, they both form a functional complex between the two-component proteins[2,3] that allow electron flow from the metal center of the reductase ($[Fe_4S_4]$ cluster) to those of the catalytic component (in the sequence of the P-cluster to the FeV or FeMo cofactors) for the eventual substrate reduction that occurs at the cofactor site (Figure 1).

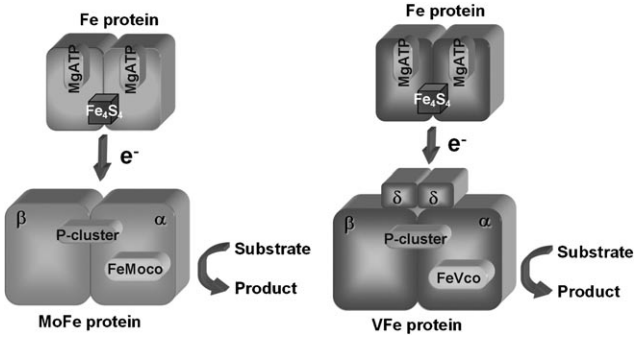

Figure 1. Schematic representation of the components of Mo and V nitrogenases. It is hypothesized that, during catalysis, the Fe protein forms a functional complex with one αβ-subunit half of the MoFe protein (left) or VFe protein (right), in which electrons are sequentially transferred from the $[Fe_4S_4]$ cluster (of the Fe protein), through the P-cluster, to the FeMo cofactor (of the MoFe protein) or the FeV cofactor (of the VFe protein), where substrate reduction eventually occurs.

Given the similarities between the V and Mo nitrogenases, it is not surprising that their catalytic profiles closely resemble each other, both covering a variety of substrates, such as N_2, protons (H^+), and acetylene (C_2H_2).[4] However, there are clear distinctions between the catalytic capacities of the two nitrogenases; most notably, the ability of V nitrogenase to utilize carbon monoxide (CO)—a well-established inhibitor of Mo nitrogenase—as a substrate for the reductive formation of small hydrocarbons: ethylene (C_2H_4), ethane (C_2H_6), and propane (C_3H_8; Figure 2).[5]

Like the reduction of N_2 to NH_3, the reduction of CO to hydrocarbons by V nitrogenase is accompanied by the

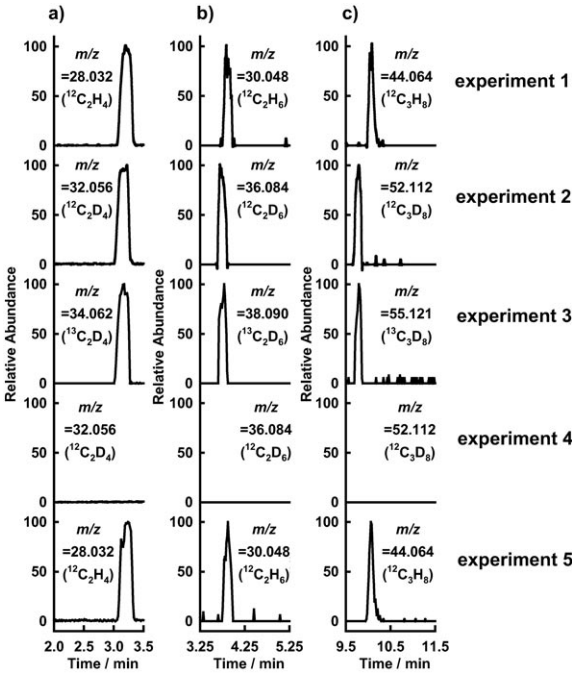

Figure 2. GC–MS analysis of a) ethylene, b) ethane, and c) propane formed by V nitrogenase. Samples were prepared in H_2O-based buffers and 100% ^{12}CO (experiment 1), D_2O-based buffers and 100% ^{12}CO (experiment 2), D_2O-based buffers and 100% ^{13}CO (experiment 3), and H_2O-based buffers and 94.5% ^{12}CO (experiment 4) plus 5.5% D_2 (experiment 5). Indistinguishable results were obtained when samples were prepared in H_2O-based buffers and 98.8% ^{12}CO plus 1.2% D_2 (data not shown). Protonated (experiments 1 and 5) and deuterated (experiments 2 and 4) products generated in the presence of ^{12}CO were traced at the following mass-to-charge (*m*/*z*) ratios: a1 and a5) 28.032, $^{12}C_2H_4$; a2 and a4) 32.056, $^{12}C_2D_4$; b1 and b5) 30.048, $^{12}C_2H_6$; b2 and b4) 36.084, $^{12}C_2D_6$; c1 and c5) 44.064, $^{12}C_3H_8$; c2 and c4) 52.112, $^{12}C_3D_8$. Deuterated products (experiment 3) generated in the presence of ^{13}CO were traced at the following mass-to-charge (*m*/*z*) ratios: a3) 34.062, $^{13}C_2D_4$; b3) 38.090, $^{13}C_2D_6$; and c3) 55.121, $^{13}C_3D_8$.

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concomitant reduction of H^+ to H_2.[5] Thus, the hydrogen used to generate the hydrocarbons could come either directly from H^+ (analogous to the nitrogenase-based N_2 reduction[1] that involves the addition of H^+ and e^- to N_2) or indirectly from H_2 (analogous to the industrial, Fischer–Tropsch-based hydrocarbon formation[6] that involves the hydrogenation of CO). Previous work showed that the formation of hydrocarbons by V nitrogenase was inhibited by the addition of increasing amounts of H_2, suggesting that H_2 was unlikely the hydrogen source for this reaction. Here, we report the results of isotope experiments for the V nitrogenase-catalyzed reduction of CO, which include the identification of the hydrogen source, as well as the detection of a new product.

As reported earlier,[5] when CO is reduced by V nitrogenase in the presence of H^+ (i.e., a H_2O-based buffer), C_2H_4, C_2H_6, and C_3H_8 can be detected by GC–MS at *m*/*z* ratios of 28.032, 30.048, and 44.064, respectively (Figure 2a–c, experiment 1). Upon substitution of H^+ by D^+ (i.e., the D_2O-based buffer), the masses of these products shift by +4, +6, and +8, respectively, consistent with the formation of C_2D_4, C_2D_6, and C_3D_8 (Figure 2a–c, experiment 2). Additional mass shifts of +2, +2, and +3 are observed when ^{13}CO is supplied together with D^+, corresponding to the formation of double-labeled products, $^{13}C_2D_4$, $^{13}C_2D_6$, and $^{13}C_3D_8$ (Figure 2a–c, experiment 3). Apart from the mass shifts, the incorporation of D^+ in these products is further demonstrated by the fact that they elute slightly faster than their respective protonated counterparts (see Figure 2a–c, experiment 1 vs. experiment 2). Such a behavior is characteristic of deuterated compounds, which usually show a decrease in the retention time on the nonpolar GC–MS columns.[7] However, when 5.5% D_2 is supplied together with H^+, no deuterated products can be detected (Figure 2a–c, experiment 4), although the formation of C_2H_4, C_2H_6, and C_3H_8 is unaffected (Figure 2a–c, experiment 5). The same effect is reproduced when 1.2% D_2 is supplied to the reaction (data not shown). Apparently, the hydrogen in the hydrocarbon does not come from the concomitant evolution of H_2 by V nitrogenase, as 1.2 and 5.5% D_2 represent the amounts of H_2 produced at 10 min and 1 h, respectively, concurrent with the hydrocarbons.[5] Together, these results firmly establish the soluble H^+ ions (rather than H_2) as the source of hydrogen for V-nitrogenase-based hydrocarbon formation.

Interestingly, when H^+ is replaced by D^+ in the reaction mixture, a new hydrocarbon product can be detected. The time-dependent formation of this product is observed in the presence of D^+, but not in the presence of H^+ (Figure 3a). GC–MS analysis further confirms the identity of this product as deuterated propylene (C_3D_6), which displays an *m*/*z* ratio of 48.084 (Figure 3b, middle). There is a further shift in the mass of this hydrocarbon product by +3 when ^{13}CO is used in combination with D^+ as the substrates, consistent with the formation of $^{13}C_3D_6$ in this reaction (Figure 3b, bottom). In contrast, C_3H_6 is not detected by GC–MS when H^+ is supplied to the reaction (Figure 3b, top). It is likely, therefore, that C_3H_6 is an intermediate that occurs during the V-nitrogenase-catalyzed extension of the hydrocarbon chain; however, it is normally not detectable because of its rapid turnover to C_3H_8 in the presence of H^+.

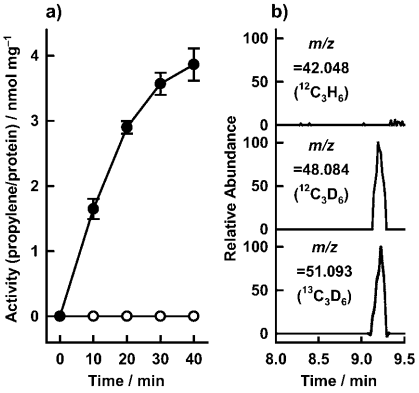

Figure 3. Formation of propylene by V nitrogenase. a) Time-dependent formation of $^{12}C_3D_6$ (●) and $^{12}C_3H_6$ (○) in the presence of 100% ^{12}CO. The samples were prepared in D_2O (●)- and H_2O (○)-based buffers. The data are presented as mean value ± standard deviation ($N=5$). b) GC–MS analysis of $^{12}C_3H_6$ (top), $^{12}C_3D_6$ (middle), and $^{13}C_3D_6$ (bottom) formed by vanadium nitrogenase. The samples were prepared in H_2O- (top) or D_2O- (middle and bottom) based buffers and contained 100% ^{12}CO (top and middle) or 100% ^{13}CO (bottom). The products were traced at the following mass-to-charge (*m*/*z*) ratios: 42.048: $^{12}C_3H_6$ (top), 48.084: $^{12}C_3D_6$ (middle), and 51.093: $^{13}C_3D_6$ (bottom).

The identification of H^+ ions as the hydrogen source for hydrocarbon formation by V nitrogenase points to a parallelism between the enzyme-based CO and N_2 reduction, as both reactions involve the ATP-dependent addition of H^+ and e^- to the substrate and the concomitant evolution of H_2 as a side product (Figure 4). Such an analogy implies some mechanistic

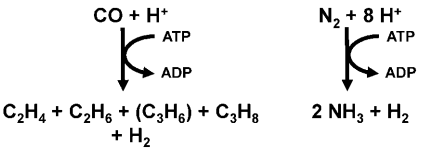

Figure 4. Proposed reaction schemes for the reduction of CO (left) and N_2 (right) by V nitrogenase. Both reactions involve the ATP-dependent protonation of substrates and the concomitant evolution of H_2.

similarities between the two reactions, particularly considering the isoelectronic properties of CO and N_2. On the other hand, the reactions of CO and N_2 reduction differ in that the former favors the reductive formation of C–C bonds from CO and the progressive extension of hydrocarbon chains, whereas the latter supports the complete cleavage of the triple bond of N_2 and the formation of fully reduced NH_3. The deuterium effect on the former reaction is especially interesting, as the solvent isotope effects of D_2O/H_2O are well-documented and can often be used to address the mechanistic questions of enzymatic reactions.[8] In the current case, the deuterium-dependent formation of C_3D_6 could be explained by inverse kinetic isotope effects (i.e., $k_H/k_D < 1$) that favor the formation of deuterated products.[9–11] However, such an effect is not consistently observed in the V-nitrogenase-catalyzed formation of other hydrocarbon products; rather, there is an

overall decrease in the amounts of hydrocarbons (by ca. 10%) and H_2 (by ca. 30%) formed in the presence of D^+ relative to those formed in the presence of H^+ (data not shown). This observation leads to an alternative explanation for the preferential formation of C_3D_6, one that involves the "stalling" of the overall reaction and the accumulation of reaction intermediates as a result of the slower incorporation of D^+ into certain hydrocarbon products. It should be noted, however, that the effect of deuterium on V-nitrogenase-catalyzed reduction of CO is likely multifaceted, and other solvent isotope effects of D_2O/H_2O, such as those affecting the protein conformation, the protein–protein interactions, and the network of hydrogen bonds, should not be overlooked.[12–15] Future investigations will combine these isotope experiments with systematic kinetic analyses, in the hope of elucidating the mechanistic details of hydrocarbon formation by V nitrogenase.

Experimental Section

Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Natural abundance ^{12}CO (99.5% purity) was purchased from Airgas (Lakewood, CA, USA). All isotope-labeled compounds (isotopic purity $\geq 98\%$) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The VFe protein and the *vnfH*-encoded Fe protein were prepared as previously described.[4]

Activity assay: Unless otherwise specified, all nitrogenase activity assays were carried out in the presence of 100% ^{12}CO or ^{13}CO at ambient temperature and pressure as described earlier.[3,4,16] All activity assays contained dithionite as the electron source. Activity analyses in D^+ were carried out by exchanging protein solutions extensively into a D_2O-based, 25 mM (D11)-Tris (i.e., $(DOCD_2)_3CND_2$) buffer and by dissolving all other components in the same buffer. The pD was adjusted to 8.0 with DCl and NaOD. Activity analyses in D_2 were carried out in the presence of 1.2 or 5.5% D_2 (with CO making up the remaining gas phase), which mimicked the concomitant evolution of H_2 by V nitrogenase at 10 min and 1 h, respectively.[5] Activity determination of the pD of this buffer was based on an established equation: pD = measured pH + 0.40[17] and further confirmed by pH indicator strips. Simultaneous determination of the hydrocarbon products was carried out on an alumina F-1 column (Grace, Deerfield, IL, USA), and the products C_2H_4/C_2D_4 C_2H_6/C_2D_6, C_3H_8/C_3D_8, and C_3H_6/C_3D_6 were analyzed and quantified as published elsewhere.[4,18]

GC–MS analysis: Samples were prepared as above, except that the reactions were terminated after 5 h. GC–MS analysis was performed using an Agilent 6890 GC 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 operated at 125°C in split mode (30:1 split ratio). Gas separation was achieved with a PLOT-Q capillary column (0.320 mm ID × 30 m length) held at 40°C for 1 min, and then heated up to 120°C at 5°C min^{-1} and held at this temperature for another 3 min. Carrier He gas was passed through the column at 1.1 mL min^{-1}. The mass spectrometer was operated in the electron-impact ionization mode at resolution of 7000 and calibrated over a range of m/z ratios between 18 and 614 using reference H_2O, N_2, O_2, Ar, and CO_2 in addition to ions from the mass reference compound tris(perfluoro-tributyl) amine. The calibrated mass axis was locked to the CF_3^+ ion at an m/z ratio of 68.995.

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