Carbon Dioxide Hot Paper

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Differential Reduction of CO_2 by Molybdenum and Vanadium Nitrogenases**

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Abstract: *The molybdenum and vanadium nitrogenases are two homologous enzymes with distinct structural and catalytic features. Previously, it was demonstrated that the V nitrogenase was nearly 700 times more active than its Mo counterpart in reducing CO to hydrocarbons. Herein, a similar discrepancy between the two nitrogenases in the reduction of CO_2 is reported, with the V nitrogenase being capable of reducing CO_2 to CO, CD_4, C_2D_4, and C_2D_6, and its Mo counterpart only capable of reducing CO_2 to CO. Furthermore, it is shown that the V nitrogenase may direct the formation of CD_4 in part via CO_2-derived CO, but that it does not catalyze the formation of C_2D_4 and C_2D_6 along this route. The exciting observation of a V nitrogenase-catalyzed C–C coupling with CO_2 as the origin of the building blocks adds another interesting reaction to the catalytic repertoire of this unique enzyme system. The differential activities of the V and Mo nitrogenases in CO_2 reduction provide an important framework for systematic investigations of this reaction in the future.*

Nitrogenases are a family of complex metalloenzymes that catalyze a key step in the global nitrogen cycle: the reduction of atmospheric nitrogen (N_2) to a bio-accessible form, ammonia (NH_3).[1–4] Apart from N_2, nitrogenases are also capable of reducing alternative substrates, such as acetylene (C_2H_2) and carbon monoxide (CO), thereby displaying a unique versatility in processing small carbon-containing molecules.[1,5] The molybdenum and vanadium nitrogenases are two homologous members of this enzyme family, sharing a good degree of homology in primary sequence and cluster composition.[5,6] Both enzymes are homologous binary systems that consist of 1) a reductase component (*nifH*- or *vnfH*-encoded Fe protein), which contains one subunit-bridging $[Fe_4S_4]$ cluster and one ATP-binding site per subunit; and 2) a catalytic component (*nifDK*-encoded MoFe or *vnfDGK*-encoded VFe protein), which contains a P-cluster at the α/β-subunit interface and a cofactor (FeMoco or FeVco) within each α-subunit (Figure 1 A). Moreover, both enzymes use the same mode of action during catalysis, which involves the formation of a functional complex between the two component proteins,[7,8] the ATP-dependent transfer of electrons from the $[Fe_4S_4]$ cluster of the reductase component to the cofactor of the catalytic component via the P-cluster, and the eventual reduction of substrates at the cofactor site upon accumulation of a sufficient amount of electrons (Figure 1 A).

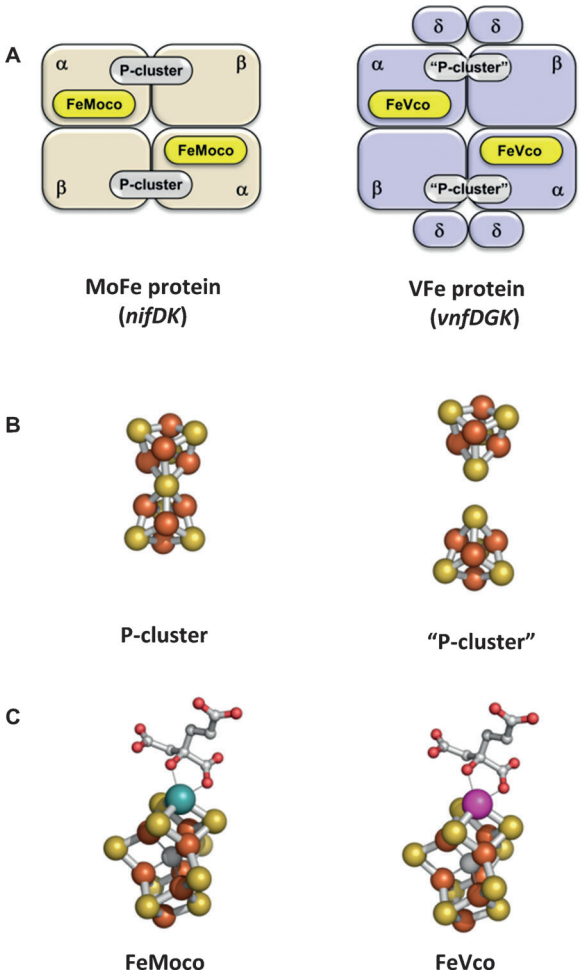

Figure 1. Comparison between the Mo and V nitrogenases. Schematic representations of the catalytic components (A) and structural models of the P-clusters (B) and cofactors (C) in the Mo (left) and V (right) nitrogenases. Carbon, gray; iron, orange; molybdenum, cyan; oxygen, red; sulfur, yellow; vanadium, magenta.

Despite their homology in structure and function, the two nitrogenases are clearly distinct from each other with regard to their associated metalloclusters. The P-cluster of the Mo nitrogenase assumes a "standard" $[Fe_8S_7]$ structure, whereas the P-cluster of the V nitrogenase consists of a pair

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of $[Fe_4S_4]$-like clusters (Figure 1B).[5,7–9] Likewise, despite a striking homology in structure, the cofactors of the Mo and V nitrogenases are distinguishable not only by the incorporated heterometals, but also by their electronic properties (Figure 1C).[10] The differences between the metal clusters in the Mo and V nitrogenases underline the differences in the catalytic behavior of these homologous enzymes. It has been documented that the V nitrogenase is less efficient than its Mo counterpart in terms of N_2 reduction; yet, this nitrogenase can reduce C_2H_2 to ethane (C_2H_6), a catalytic activity not observed in the case of the Mo nitrogenase.[6,8] Perhaps the biggest discrepancy between the catalytic properties of the two nitrogenases is their ability to reduce CO to hydrocarbons, with the V nitrogenase showing an overall activity that is nearly 700 times higher than that of its Mo counterpart.[11,12] This observation has prompted us to conduct a comparative study with the Mo and V nitrogenases to address 1) whether the two nitrogenases can also reduce CO_2 to hydrocarbons, and 2) whether they have the same discrepancy in their activities to generate hydrocarbons from this substrate.

Consistent with an earlier report,[13] the Mo nitrogenase can reduce CO_2 to CO (Figure 2A, triangles). Like its Mo counterpart, the V nitrogenase can also catalyze the reduction of CO_2 to CO (Figure 2A, circles) in an ATP-dependent reaction (Figure S1) using dithionite (20 mM) at pH 8.5. The two nitrogenases displayed comparable efficiencies in H_2O-based reactions, forming approximately the same amount of CO from CO_2 over a time period of 180 minutes (Figure 2A). Moreover, both nitrogenases exhibited roughly the same increase in activity for the formation of CO from CO_2 upon substitution of D_2O for H_2O, reaching a maximum increase of activity at 120 minutes (Figure 2A). Apart from CO, CH_4, which is a further reduced C_1 product, could be detected in reaction mixtures in the presence of the Mo and V nitrogenases when CO_2 was supplied as a substrate (Figure 2B). However, when H_2O was replaced by D_2O, the activity of CH_4 formation by the V nitrogenase increased from 0 to a maximum of 22.2 nmol per μmol of protein (Figure 2B, ● vs. ○), whereas the activity of CH_4 formation by the Mo nitrogenase decreased from a maximum of 7.3 nmol per μmol of protein to 0 (Figure 2B, ▼ vs. ▽). Such a disparate D_2O effect implies a difference in the routes to CH_4 formation taken by the two nitrogenases.

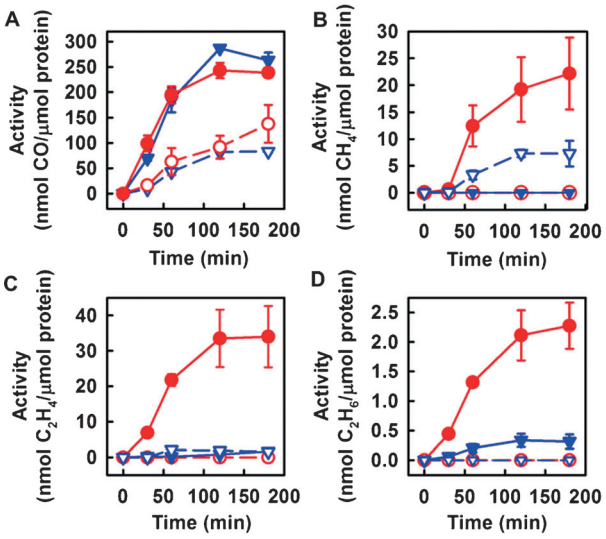

Figure 2. Product formation by the Mo and V nitrogenases in the presence of CO_2. Time-dependent formation of CO (A), CH_4 (B), C_2H_4 (C), and C_2H_6 (D) by the Mo nitrogenase in H_2O (▽, – – –) or D_2O (▼, ——) and by the V nitrogenase in H_2O (○, – – –) or D_2O (●, ——). Data are presented as mean ± SD ($N = 3$) after background correction.

The difference between the V and Mo nitrogenases in CO_2 reduction is further illustrated by the difference in their abilities to use CO_2 as a substrate to form C–C bonds. In the presence of H_2O, little or no C_2 product was detected during CO_2 reduction by either the Mo or the V nitrogenase (Figure 2C and D, ▽ and ○). In the presence of D_2O, however, C_2D_4 (Figure 2C, ●) and C_2D_6 (Figure 2D, ○) were detected as products of CO_2 reduction by the V nitrogenase, whereas these C_2 products were hardly detectable in the same reaction catalyzed by the Mo nitrogenase (Figure 2C and D, ▼). Thus, as was observed in the case of CH_4 formation, there was a clear increase in the activities of C_2D_4 and C_2D_6 formation by the V nitrogenase upon substitution of D_2O for H_2O, whereas these activities remained marginal in the reaction catalyzed by the Mo nitrogenase following such a substitution. Moreover, like the formation of CH_4, the formation of C_2 products by the V nitrogenase was ATP-dependent, as C_2D_4 and C_2D_6 could not be detected in the absence of ATP (Supporting Information, Figure S1).

GC–MS analysis supplied further evidence for the differences between the Mo and V nitrogenases in hydrocarbon formation from CO_2. When $^{12}CO_2$ was replaced by $^{13}CO_2$, $^{13}CD_4$ could be detected in the V nitrogenase-catalyzed reaction in D_2O (Figure 3B); however, $^{13}CH_4$ was absent from the Mo nitrogenase-catalyzed reaction in H_2O (Figure 3A). This observation confirmed CO_2 as the carbon source for CD_4 generated by the V nitrogenase while suggesting a different carbon source for the same C_1 product generated by the Mo nitrogenase. Aside from CD_4, CO_2 also gave rise to the C_2 products in the V nitrogenase-catalyzed reaction, as $^{13}C_2D_4$ (Figure 3C) and $^{13}C_2D_6$ (Figure 3D) could be detected in the presence of D_2O upon substitution of $^{13}CO_2$ for $^{12}CO_2$. Together, the GC–MS and activity data highlight the difference between the reactions of CO_2 reduction by the V and Mo nitrogenases, showing the ability of the V nitrogenase to form C_1 and C_2 hydrocarbons along with CO and the inability of its Mo counterpart to generate products other than CO under these experimental conditions. Given the previous observation that the V nitrogenase can reduce CO to hydrocarbons,[11,12] the co-production of CO and hydrocarbons by this enzyme as products of CO_2 reduction raises the relevant question of whether it is the CO_2-derived CO that gives rise to the hydrocarbon products.

This question can be addressed by directly supplying CO to the V nitrogenase in a concentration simulating the maximum concentration of CO achieved in the "equilibrated state" of CO_2 reduction by this enzyme (see Figure 2A) and monitoring the formation of the C_1 and C_2 hydrocarbons in D_2O over a time period of 180 minutes. Interestingly, the CO-based formation of CD_4 by the V nitrogenase (Figure 4A, ○)

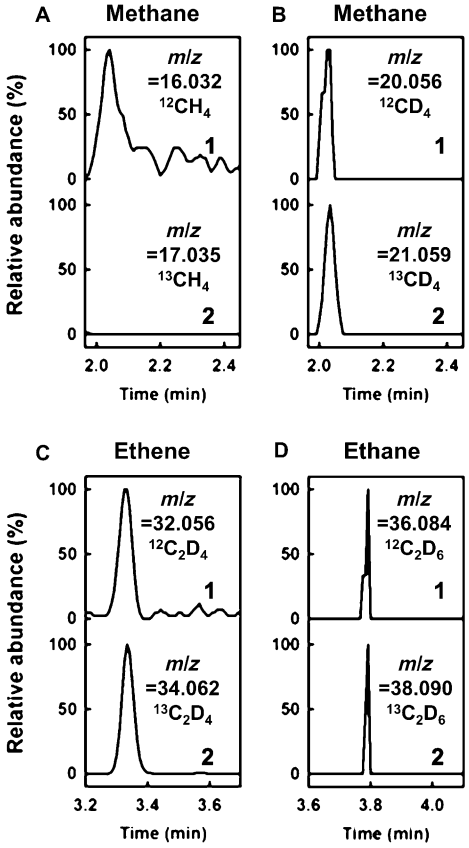

Figure 3. GC–MS analyses of the hydrocarbon products formed by the Mo and V nitrogenases. The products were generated by the Mo nitrogenase in H_2O (A) or by the V nitrogenase in D_2O (B–D) when $^{12}CO_2$ (**1**) or $^{13}CO_2$ (**2**) was supplied. The mass-to-charge (m/z) ratios at which the products were traced are indicated.

displayed an activity increase of 12.6 nmol per μmol of protein between 0 and 30 minutes, whereas the CO_2-based formation of CH_4 exhibited a nearly identical activity increase of 11.8 nmol per μmol of protein between 30 and 60 minutes after an initial lag phase between 0 and 30 minutes (Figure 4A, ●). This observation suggests the possibility for the V nitrogenase to direct the formation of C_1 hydrocarbons via CO, as the 30-minute delay could be correlated with a need for the enzyme to accumulate a sufficient amount of CO_2-derived CO to initiate further reduction of CO to CD_4. On the other hand, the time courses of CD_4 formation from CO (Figure 4A, ○) and CO_2 (Figure 4A, ●) diverged beyond 60 minutes, with a gradual decrease in activity for the former and a gradual increase in activity for the latter. The difference between the two time courses (Figure S2A) could represent the portion of CD_4 that is generated independently from CO_2-derived CO. Consistent with this hypothesis, there is a notable difference between the CO- and CO_2-based reactions in the percentage activity of CH_4 formation in H_2O relative to that in D_2O (Figure S3A), with the CO-based reaction favoring the formation of CH_4 in H_2O over that in D_2O (42%) considerably more than the CO_2-based reaction (0%). It is therefore possible that the V nitrogenase generates CH_4 both from CO_2-derived CO and from CO_2 and/or other CO_2-derived intermediate(s).

Unlike CD_4, both C_2D_4 and C_2D_6 seem to be produced by the V nitrogenase along a CO-independent route, as no C_2 products could be detected (Figure 4B and C, ○) upon direct addition of the same amount of CO as produced by the V nitrogenase through CO_2 reduction in the equilibrated state (see Figure 2A). This observation suggests that instead of CO, CO_2 and/or other CO_2-derived intermediates are responsible for the formation of C_2 hydrocarbon products by the V nitrogenase. Indeed, as was observed in the case of CH_4 formation, there is a significant difference between the CO- and CO_2-based reactions in the percentage activity of C_2H_4 (Figure S3B) or C_2H_6 (Figure S3C) formation in H_2O relative to that in D_2O, with the CO-based reaction favoring the formation of C_2 products in H_2O over that in D_2O (C_2H_4, 92%; C_2H_6, 65%) considerably more than the CO_2-based reaction (C_2H_4, 0.7%; C_2H_6, 0%). Such a disparate deuterium effect on the CO- and CO_2-based reactions further implies that the V nitrogenase directs the formation of C_2 hydrocarbons via CO_2 or other CO_2-derived intermediate(s). The lack of contribution of CO to the formation of C_2 hydrocarbons in this case could be explained by an insufficient CO concentration achieved by the reduction of CO_2, which does not allow the formation of C–C bonds. More excitingly, it defines the ability of the V nitrogenase to directly use CO_2 as a substrate for the initial C–C coupling and the subsequent carbon chain extension.

The ability of certain variants of the Mo nitrogenase to reduce CO_2 to CH_4 was reported recently.[14] To our surprise, contrary to what has been described for these variants of the Mo nitrogenase, the wild-type Mo nitrogenase cannot reduce CO_2 to CH_4; rather, it uses an unknown carbon source to generate CH_4 in the presence of CO_2 and H_2O. Considering the presence of an interstitial carbide[15–18] and a homocitrate moiety in the FeMoco,[15,17] it can be postulated that in H_2O, CO_2 or its derivative somehow promotes the release of the central carbide ligand or carbon-containing groups of the homocitrate in the form of CH_4. Alternatively, the side-chain groups of certain amino acids at the active site of the Mo ni-

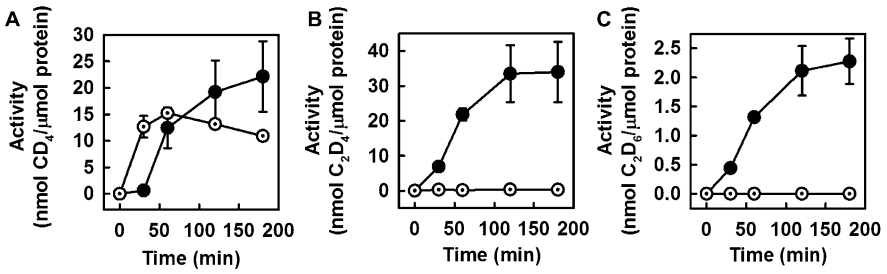

Figure 4. Formation of hydrocarbon products by the V nitrogenase. Time-dependent formation of CD_4 (A), C_2D_4 (B), and C_2D_6 (C) from CO_2 (●) or CO (○) by the V nitrogenase in D_2O. CO was added at a concentration of 110 ppm in assays involving the direct formation of products from CO, which was equivalent to the maximum concentration of CO that could be generated from CO_2 reduction by the V nitrogenase (see also Figure 2). Data are presented as mean ± SD ($N = 3$) after background correction.

trogenase may also serve as a carbon source for the production of CH_4 in the presence of CO_2. Remarkably, despite the unclear nature of the carbon source, the formation of CH_4 by the Mo nitrogenase is ATP-dependent and requires the presence of both component proteins; moreover, it only occurs in the presence of CO_2 and H_2O (Figure S4). This observation points to a redox-dependent mechanism for this reaction, as the requirement for ATP and both components is specifically associated with the transfer of electrons through the enzyme system, which may permit the initial binding and processing of CO_2 or its derivative in H_2O and the subsequent interaction between CO_2 or CO_2-derived intermediate(s) and the carbon species that eventually gives rise to CH_4. Given the overall homology between the Mo and V nitrogenases, one would expect the V nitrogenase to catalyze the same unspecific formation of CH_4 from a different carbon source than CO_2 as its Mo counterpart. Although this possibility cannot be ruled out, our current data (see Figure 3 A and B) clearly demonstrate that the CH_4 formed by the V nitrogenase is derived, at least in part, from CO_2. Further investigations of the origin of the different routes taken by the two nitrogenases to CH_4 formation could be informative, particularly with regard to the initial binding and processing of CO_2 by this enzyme system.

Based on the hydrocarbon products identified thus far in the gas phase, the V nitrogenase generates carbon-containing compounds at a slow rate from CO_2 reduction, forming 0.3 mol CO, 0.02 mol CH_4, 0.04 mol C_2H_4, and 0.002 mol C_2H_6 per mol of protein. Nevertheless, the ability of the V nitrogenase to form hydrocarbons, particularly the C_2 products, from CO_2, is a most remarkable finding of the current study, because it adds another exciting reaction to the catalytic repertoire of this unique enzyme system. As was observed in the case of CO reduction,[12] the V nitrogenase is superior to its wild-type Mo counterpart in generating hydrocarbons from CO_2. The disparate CO-reducing activities of the V and Mo nitrogenases were compared with the differential capacities of synthetic V and Mo compounds to reductively couple two CO moieties into functionalized acetylene ligands.[19] An alteration of the CO-reducing activities was reported for the MoFe protein variants that contained modified residues at the active site.[20] By analogy, the disparate CO_2 reducing activities of the two nitrogenases could also stem from the structural/redox differences between FeVco and FeMoco, as well as the protein environments surrounding the two cofactors (see Figure 1). Furthermore, the different structural/redox properties of the P-clusters in the two nitrogenases could further contribute to the differences between their abilities to reduce CO_2 (see Figure 1). In fact, the ability of the nitrogenases to generate hydrocarbons from CO_2 was first described for a cofactor-deficient variant of the MoFe protein[21] and attributed to its unique P-cluster, which contains a $[Fe_4S_4]$-like cluster pair instead of the normal $[Fe_8S_7]$ P-cluster.[22] Interestingly, the P-cluster of the V nitrogenase also consists of a pair of $[Fe_4S_4]$-like clusters[5,8,9] and could, in principle, serve as a site for CO_2 reduction on its own; only in the case of the holo form of the V nitrogenase, the presence of the cofactor "downstream" of the P-cluster along the electron transfer pathway (see Figure 1) may effectively "funnel" the electrons towards the cofactor site and only allow a small amount of CO_2 reduction at the P-cluster site. The possibility of two reactive sites (i.e., P-cluster and cofactor) and different reaction routes (i.e., via CO or other CO_2-derived intermediates) for CO_2 reduction makes it a challenging task to elucidate the mechanistic details of this reaction. Nevertheless, the work reported herein provides an important framework for systematic investigations of this unique reaction in the future, which will hopefully lead to the development of nitrogenase-based strategies to recycle the greenhouse gas CO_2 into useful carbon fuel.

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