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Evaluation of the Catalytic Relevance of the CO-bound States of V-Nitrogenase

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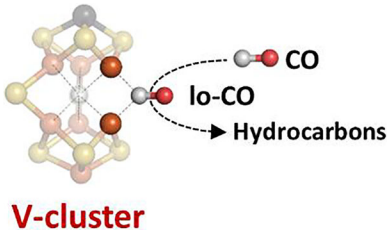
Abstract

Binding and activation of CO by nitrogenase is a topic of interest because CO is isoelectronic to N_2, the physiological substrate of this enzyme. Here we examine the catalytic relevance of one- and multi-CO-bound states (i.e., the lo- and hi-CO states) of V-nitrogenase to C-C coupling and N_2 reduction. Enzymatic and spectroscopic studies demonstrate that the multiple CO moieties in the hi-CO state cannot be coupled as they are, suggesting that C-C coupling requires further activation and/or reduction of the bound CO entity. Moreover, these studies reveal an interesting correlation between decreased activity of N_2 reduction and increased population of the lo-CO state, pointing to the catalytic relevance of the 'belt Fe' atoms that are bridged by the single CO moiety in the lo-CO state. Together, these results provide a useful framework for gaining insights into the nitrogenase-catalyzed reaction via further exploration of the utility of the lo-CO conformation of V-nitrogenase.

Graphical Abstract

The multiple CO moieties captured in the hi-CO state of V-nitrogenase cannot be coupled as they are. Instead, the single CO moiety in the lo-CO state is competent in C-C coupling with externally supplied CO molecules. Moreover, an increase in the population of the lo-CO state correlates with a decrease in the activity of N_2 reduction, suggesting a catalytic relevance of the 'belt Fe' atoms that are bridged by CO in the lo-CO state.



Keywords

nitrogenase; vanadium; cofactor; CO binding; CO reduction

Nitrogenase is a versatile metalloenzyme that is capable of ambient reduction of a wide range of small molecules.[1] Utilizing a reductase component to deliver electrons to its catalytic component, nitrogenase is best known for its function in reducing N_2 to NH_4^+ in a process called biological nitrogen fixation, which predates and parallels the industrial Haber-Bosch process.[2] Recently, this enzyme was also shown to reduce CO to hydrocarbons[3,4] in a reaction that mirrors the industrial Fischer-Tropsch process,[5,6] further highlighting its importance in energy- and environment-related areas. Binding and activation of CO by nitrogenase has been a topic of vigorous research because CO is isoelectronic to N_2. Interestingly, despite their overall similarities (Figure S1),[7–11] the homologous, wildtype Mo- and V-nitrogenases display differential reactivities toward CO, with the former capable of binding CO but nearly inactive in CO reduction and the latter showing an activity of 16.5 nmol reduced C/nmol protein/min in reducing CO to hydrocarbons.[4,12,13] The disparate CO reactivities of these homologous nitrogenases suggest the possibility to investigate the activity of V-nitrogenase in CO reduction by drawing comparisons between V-nitrogenase and its Mo-counterpart in their interactions with CO. Previously, we generated a one CO-bound form of the resting-state VFe protein (the catalytic component of V-nitrogenase) in the presence of Eu^{II}-DTPA, which displayed an EPR signal resembling that of the one-CO-bound (i.e., the lo-CO state) MoFe protein (the catalytic component of Mo-nitrogenase), although the latter could only be generated under turnover conditions.[9] This conformation of VFe protein was subsequently assigned as one with CO bridged between a pair of Fe atoms across the ‘belt’ of the cofactor based on its homology to the one-CO-bound MoFe protein (Figure S2a, *left*).[9,14] However, contrary to the lo-CO conformation of the MoFe protein, the lo-CO conformation of the VFe protein was capable of turning over the bound CO molecule, generating C1 and C2 hydrocarbon products, respectively, in the absence and presence of ‘extra’ CO.[9] Success in generating such a catalytically competent, one CO-bound state of the VFe protein has prompted us to seek conditions to trap more than one CO molecule on this protein (i.e., the hi-CO state) for investigations of C-C coupling by V-nitrogenase.

To generate a multi-CO-bound state, the resting-state VFe protein of *A. vinelandii* (designated $VnfDGK^{Av}$) was first incubated with CO at an overpressure of 2.6 atm in the presence of a strong reductant, europium(II) diethylenetriaminepentaacetic acid (Eu^{II}-DTPA; $E^{0'}$=−1.14 V at pH 8),[15,16] and then re-isolated into a reductant-free buffer under 1 atm Ar. Compared to the one-CO-bound conformation of $VnfDGK^{Av}$ that was generated by the

same procedure under 1 atm CO (Figure 1a, *1*), VnfDGK^{Av} generated under over-pressurized CO not only displayed EPR features (g = 2.09, 1.99 and 1.91) of the one-CO-bound (lo-CO) conformation, but also showed additional EPR features (g = 2.13, 2.01 and 1.97) that originated from the capture of 'extra' CO molecules on the protein (Figure 1b, *1*). Interestingly, the major features of the lo-CO spectrum and the 'extra-CO' spectrum (i.e., the difference spectrum between the multi-CO- and one-CO-bound spectra) could be approximated by simulating a single species for each spectrum (Figure 1a, *2;* Figure 1c, *2*); whereas the features of the unsubtracted, multi-CO-bound (hi-CO) spectrum could be approximated by summation of the lo-CO and extra-CO components (Figure 1b, *2*). However, there were additional fine structures that could not be resolved based on these CW EPR data.

Quantification of CO that was bound to VnfDGK^{Av} was performed by acid quench and revealed the presence of 1.0 ± 0.1 and 3.7 ± 0.2 mol bound CO/mol protein, respectively, in the lo-CO and hi-CO states (Figure 1d, *3, 4*). The inability of the cofactor-deficient, apo VnfDGK to capture any CO under the same experimental conditions used to generate the lo- and hi-CO states (Figure 1d, *1*, *2*; *also see* Figure S3) pointed strongly to the cofactor (V-cluster) as the site of CO binding. Notably, an earlier stopped-flow FTIR study of the *A. vinelandii* MoFe protein (designated NifDK^{Av}) reported observation of a single absorption at low CO concentrations and 3−4 absorptions at high CO concentrations,[17] which were in line with our results of VnfDGK^{A}—the counterpart of NifDK^{Av} in the V-nitrogenase system. Moreover, the 'extra-CO' EPR spectrum of VnfDGK^{Av} (Figure 1c, *1*) loosely resembled the previously reported spectrum of NifDK^{Av} that was bound with neighboring CO molecules, suggesting the presence of adjacently bound CO molecules in the multi-CO-bound VnfDGK^{Av} like what was described in the case of the multi-CO-bound NifDK^{Av}.[18,19] Given the spectral analogy between VnfDGK^{Av} and NifDK^{Av} in CO binding, the multi-CO-bound, hi-CO state of VnfDGK^{Av} could assume a conformation analogous to the one that was suggested for its NifDK^{Av} counterpart,[20–22] with at least two CO molecules bound side by side to a pair of Fe atoms across the S belt of the cofactor (Figure S2a, *right*).

Interestingly, upon reduction of the electron flux, the same hi-CO conformation of VnfDGK^{Av} could be observed under turnover conditions, when VnfDGK^{Av} was combined with the Fe protein (the reductase component of V-nitrogenase), ATP and dithionite.[22] Reduction of the electron flux was enabled by replacing the *vnfH*-encoded Fe protein of *A. vinelandii* (designated VnfH^{Av}) with the *vnfH*-encoded Fe protein homolog of *Methanosarcina acetivorans* (designated VnfH^{Ma}),[23] which resulted in a V-nitrogenase hybrid with decreased substrate-reducing activities (Figure 2a, *1 vs. 2*). Calculations of the total amount of electrons that appeared in the products revealed an average reduction of electron flux by ~94% through the VnfH^{Ma}/VnfDGK^{Av} hybrid (Figure 2b, *2*, *left*) relative to that through the native VnfH^{Av}/VnfDGK^{Av} complex, although the most drastic decrease of electron flux was observed when CO was supplied as the substrate (Figure 2b, *2*, *right*). Strikingly, the EPR signal of the CO-bound VnfDGK^{Av} that was generated under turnover conditions (i.e., in the presence of VnfH^{Ma}) at 1 atm CO (Figure 2c, *1*) assumed a line-shape that was nearly indistinguishable from the EPR spectrum of the hi-CO state of VnfDGK^{Av} generated with the resting state protein (i.e., in the absence of VnfH^{Ma}) at 2.6 atm CO (Figure 1b, *1*). Thus, a dramatically reduced electron flux seemed to have enabled binding of

CO to the cofactor of VnfDGK^{Av} at site(s) with low affinity to CO, a feat that could only be accomplished otherwise under over-pressurized CO. This observation firmly established the relevance of the hi-CO state of VnfDGK^{Av} generated in the resting state (designated hi-CO^{A}; Figure 1b, *1*) to that generated under limited turnover conditions (designated hi-CO^{B}; Figure 2c, *1*), thereby providing two useful probes into the mechanistically-relevant questions of (i) whether the hi-CO conformation is catalytically competent in C-C coupling and (ii) which CO-bound state specifically impacts the reaction of N_2 reduction.

To address the catalytic relevance of the hi-CO conformation to C-C coupling, hi-CO^{A} was subjected to turnover conditions upon incubation with VnfH^{Av}, ATP and dithionite in the absence or presence of 'extra' CO, and examined for C-C coupling (i.e., formation of C2 products) during this process. Interestingly, C-C coupling was not detectable upon turnover of hi-CO^{A} without additional CO, as no C2 product was formed under this condition (Figure 3a, *1*; Figure S2c); however, it could be detected in the form of C_2H_4 and C_2H_6 in small quantities when hi-CO^{A} was subjected to turnover under 0.02 atm CO (Figure 3a, *2*), a CO concentration that was 1:1 in molar ratio to the concentration of cofactor that was bound to the hi-CO^{A} state of VnfDGK^{Av}. Remarkably, the amount of C2 products generated by hi-CO^{A} did not exceed that by the lo-CO state of VnfDGK^{Av} upon turnover in the presence of an equimolar amount of externally supplied CO (Figure 3a, *2 vs. 3*), suggesting that the 'extra' CO in hi-CO^{A}—which could have contributed to an increased yield of C2 products upon turnover—was not involved in C-C coupling; instead, only the CO moiety in the lo-CO conformation was catalytically competent, and it was this CO moiety that participated in C-C coupling with the CO molecule supplied in addition in the headspace. Isotope labeling experiments further demonstrated that, when hi-CO^{A} was generated with ^{13}CO and subsequently turned over under 0.02 atm ^{12}CO, the C2 products generated in this process contained mixed $^{12}C/^{13}C$ labels (i.e., $^{13}CH_2$=$^{12}CH_2$ and $^{13}CH_3$-$^{12}CH_3$) instead of a single ^{13}C label (i.e., $^{13}CH_2$=$^{13}CH_2$ and $^{13}CH_3$-$^{13}CH_3$) (Figure 3b, c; also see Figure S2d), providing additional support for the coupling between a bound carbon (i.e., a labeled ^{13}C atom) and an externally supplied carbon (i.e., an unlabeled ^{12}C atom) instead of between two bound ^{13}C atoms. Together, these observations strongly suggested that C-C coupling did not occur between the CO moieties that are trapped on the V-cluster in the hi-CO state, where some CO molecules are likely bound in a side-by-side manner (Figure S2a, *right*); instead, it took place between the carbon in the lo-CO conformation and a second carbon that was supplied externally in the headspace under turnover conditions (Figure S2d).

To address the impact of CO binding on N_2 reduction, VnfDGK^{Av} was subjected to turnover conditions in the presence of a fixed concentration of N_2 (0.1 atm) and increasing concentrations of CO (0.1, 0.5 and 0.9 atm), and monitored for changes in its activity to reduce N_2 to NH_3 concomitant with the appearance of the one-CO and extra-CO EPR features in thus-generated hi-CO^{B} state. Consistent with the well-established competition between N_2 and CO,[1] the activity of NH_3 formation decreased in the presence of increasing concentrations of CO (Figure 4a), and this decrease was accompanied by an increase in the intensities of the CO-related EPR features (Figure 4b); in particular, the g=2.09 and g=2.13 features, which did not overlap with other CO-originated signals, were used as markers to monitor the change of one- and extra-CO features, respectively, in this process (Figure 4b). Interestingly, while the decrease of N_2 reduction correlated well with the intensity gain of

the one-CO feature at g=2.09 (Figure 4c), the extra-CO feature at g=2.13 did not appear until the N_2-reducing activity was mostly abolished (Figure 4d). The fact that there is no inflection in the N_2-reducing activity curve as the intensity of the extra-CO feature increases suggests that binding and reduction of N_2 occurs independently from binding of additional CO molecules. The sharp decay of the N_2-reducing activity concomitant with a significant increase of the intensity of the one-CO feature, on the other hand, implies that N_2 and CO may compete for the same pair of reactive Fe sites across the S belt of the cofactor, although an indirect impact of CO binding on N_2 reduction—such as a change in the redox or structural properties of the cofactor upon CO binding—cannot be excluded.

Binding of CO to the wildtype Mo-nitrogenase, as well as reduction of CO by certain Mo-nitrogenase variants, has been reported previously;[14,17–22,24–27] however, the catalytic mechanism of CO reduction has remained elusive. Consistent with the DFT calculation-predicted pathway of CO reduction,[28] the current study reveals that the multiple CO moieties in the hi-CO conformation cannot be coupled as they are. Further, it provides the initial biochemical evidence for the potential relevance of the belt Fe atoms to N_2 reduction, which aligns well with results of the previous spectroscopic and structural analyses of CO binding.[14,29,30] Further exploration of the utility of the catalytically competent, CO-bound conformation of V-nitrogenase, along with H/D exchange experiments and a more extensive EPR study aiming to unveil the fine structure of the CO-bound state, is currently underway in hopes of gaining relevant insights into the catalytic mechanism of CO reduction by nitrogenase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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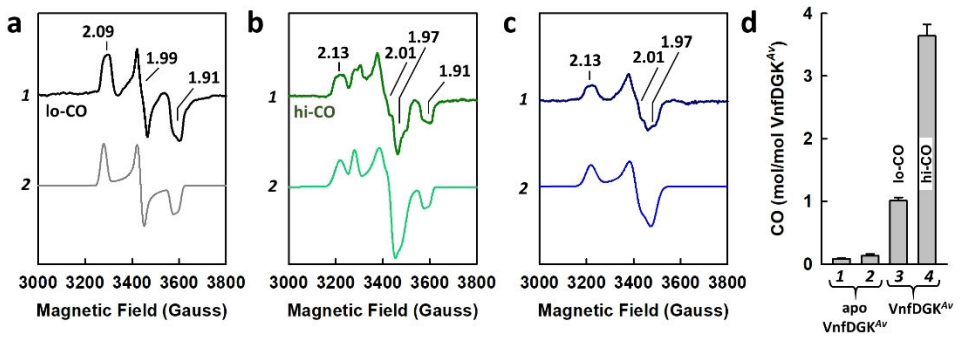

Figure 1.

EPR spectra of (**a**) the one-CO-bound (lo-CO) state generated in the presence of 1 atm CO (*1, black trace*) and (**b**) the multi-CO-bound (hi-CO) state generated in the presence of 2.6 atm CO (*1, dark green trace*). Both states were generated with the resting-state VnfDGK^{Av} (*i.e.*, without the Fe protein, the reductase partner of VnfDGK^{Av}) using Eu^{II}-DTPA as a reductant. (**c**) The difference spectrum between the hi-CO (b, *1*) and lo-CO state (a, *1*) reflects features generated upon binding of 'extra' CO molecules (*1, gray trace*). The *g* values are indicated in the figure. Spectra (a, *1*), (b, *1*) and (c, *1*) were simulated as described in the *Experimental Section* and are shown as traces (a, *2*), (b, *2*) and (c, *2*) below the corresponding spectra. (**d**) Quantification of the amounts of VnfDGK^{Av}-bound CO in the lo-CO (*3*) and hi-CO (*4*) states. The amount of bound CO was quantified upon acid quench of the hi- or lo-CO states of VnfDGK^{Av}. The same experiment was conducted on the V-cluster-deficient, apo VnfDGK^{Av}, which was incapable of capturing CO under the conditions described for the formation of the lo-CO (*1*) and hi-CO (*2*) states.

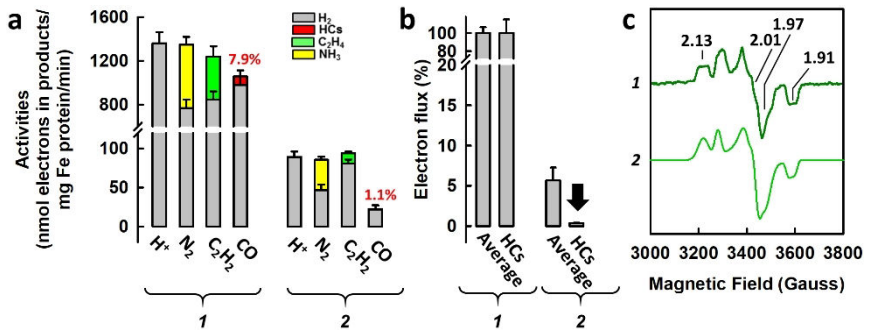

Figure 2.

(**a**) Substrate-reducing activities of VnfDGK^{Av} with VnfH^{Av} (*1*) and VnfH^{Ma} (*2*) as the respective electron donors. The activities are calculated based on the amounts of electrons that are required for the formation of the products. Substrates and products are indicated in the figure. Percentage activities of hydrocarbon formation in the presence of CO are shown in red fonts. HCs, hydrocarbons. (**b**) Average electron fluxes of all substrate-reducing reactions (*left*) and the electron fluxes of the reactions of hydrocarbon formation from CO reduction (*right*) by VnfDGK^{Av}, with VnfH^{Av} (*1*) and VnfH^{Ma} (*2*) as the respective electron donors. (**c**) EPR spectra of the turnover samples of VnfDGK^{Av} with VnfH^{Ma} (*1*) as the electron donor in the presence of 1 atm CO. The *g* values are indicated in the figure. Spectrum *2* was simulated as described in the *Experimental Section* and is shown below the corresponding spectrum *1*.

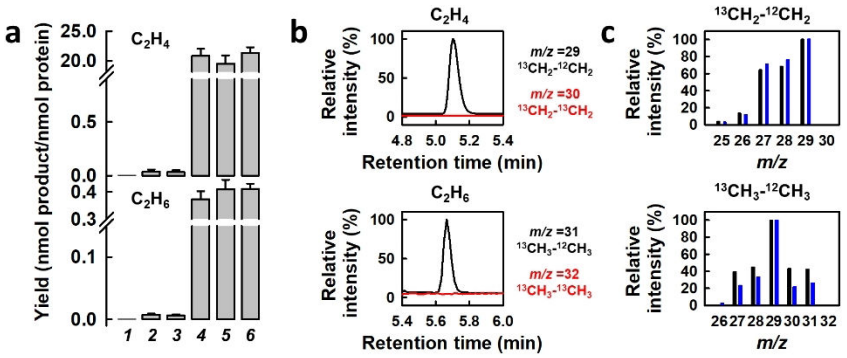

Figure 3.

Relevance of the CO-bound states of VnfDGK^{Av} to C-C coupling. (**a**) Formation of C_2H_4 (*upper*) and C_2H_6 (*lower*) by placing (*1*) hi-CO^A of VnfDGK^{Av} under turnover with no additional CO, (*2*) hi-CO^A of VnfDGK^{Av} under turnover with 0.02 atm CO, (*3*) lo-CO of VnfDGK^{Av} under turnover with 0.02 atm CO, (*4*) hi-CO^A of VnfDGK^{Av} under turnover with 1 atm CO, (*5*) lo-CO of VnfDGK^{Av} under turnover with 1 atm CO, and (*6*) VnfDGK^{Av} under turnover with 1 atm CO. All activities were measured upon incubation with VnfH^{Av}, ATP and dithionite. The observation that hi-CO^A (*4*) and lo-CO (*5*) generated approximately the same yields of the C2 products as VnfDGK^{Av} upon direct turnover of CO (*6*) in the presence of 1 atm CO demonstrated that the hi-CO^A and lo-CO species were as turnover-capable as the CO-free VnfDGK^{Av}. (**b**) GC-MS analysis of C_2H_4 (*upper*) and C_2H_6 (*lower*) that were generated by placing the ^{13}CO-labeled hi-CO^A under 0.02 atm ^{12}CO (i.e., the same turnover experiment as that in a, *2*, except for the ^{13}C labeling of hi-CO^A in this case). Both C2 products were traced at masses that would either contain mixed ^{12}C/^{13}C labels (*black traces*) or single ^{13}C isotope label (*red traces*). (**c**) GC-MS fragmentation patterns of the mixed ^{12}C/^{13}C labeled C_2H_4 (*upper, black*) and C_2H_6 (*lower, black*). The corresponding fragmentation patterns of the respective standards (*blue*) are presented based on the information from the NIST MS database (http://webbook.nist.gov). The intensities of the base peaks are set at 100% in all panels.

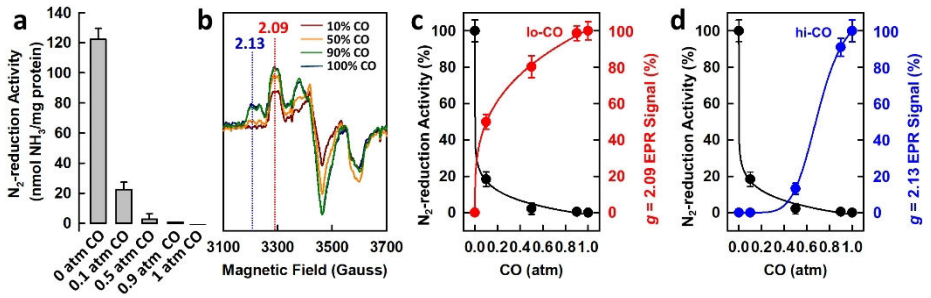

Figure 4.

Relevance of the CO-bound states of VnfDGK^{Av} to N_2 reduction. (**a**) Activities of N_2 reduction and (**b**) corresponding EPR spectra of the turnover samples in the presence of 0.1 atm N_2 and increasing CO concentrations of 0.1, 0.5 and 0.9 atm. The *g* values are indicated in the figure. Correlation of the activity of N_2 reduction with the intensity of (**c**) lo-CO or (**d**) hi-CO^{B} EPR signal in the presence of increasing CO concentrations. The EPR features at *g*=2.09 and *g*=2.13 were integrated and used as markers for the lo-CO and hi-CO^{B} signals, respectively, as they had nearly no overlap with all other CO-associated EPR features. The activity of N_2 reduction in the absence of CO, as well as the EPR signal intensities at *g*=2.09 and *g*=2.13 in the presence of 1 atm CO, are set as 100% (c, d).