Azotobacter vinelandii Vanadium Nitrogenase: Formaldehyde Is a Product of Catalyzed HCN Reduction, and Excess Ammonia Arises Directly from Catalyzed Azide Reduction†

Karl Fisher, Michael J. Dilworth,‡ and William E. Newton*

Department of Biochemistry, The Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received July 19, 2005; Revised Manuscript Received February 3, 2006

ABSTRACT: The Mo-nitrogenase-catalyzed reduction of both cyanide and azide results in the production of excess NH_3, which is an amount of NH_3 over and above that expected to be formed from the well-recognized reactions. Several suggestions about the possible sources of excess NH_3 have been made, but previous attempts to characterize these reactions have met with either limited (or no) success or controversy. Because V-nitrogenase has a propensity to release partially reduced intermediates, e.g., N_2H_4 during N_2 reduction, it was selected to probe the reduction of cyanide and azide. Sensitive assay procedures were developed and employed to monitor the production of either HCHO or CH_3OH (its further two-electron-reduced product) from HCN. Like Mo-nitrogenase, V-nitrogenase suffered electron-flux inhibition by CN^- (but was much less sensitive than Mo-nitrogenase), but unlike the case for Mo-nitrogenase, MgATP hydrolysis was also inhibited by CN^-. V-Nitrogenase also released more of the four-electron-reduced intermediate, CH_3NH_2, than did Mo-nitrogenase. At high NaCN concentrations, V-nitrogenase directed a significant percentage of electron flux into excess NH_3, and under these conditions, substantial amounts of HCHO, but no CH_3OH, were detected for the first time. With azide, in contrast to the case for Mo-nitrogenase, both total electron flux and MgATP hydrolysis with V-nitrogenase were inhibited. V-Nitrogenase, unlike Mo-nitrogenase, showed no preference between the two-electron reduction to N_2-plus-NH_3 and the six-electron reduction to N_2H_4-plus-NH_3. V-Nitrogenase formed more excess NH_3, but reduction of the N_2 produced by the two-electron reduction of N_3^- was not its source. Rather, it was formed directly by the eight-electron reduction of N_3^-. Unlike Mo-nitrogenase, CO could not completely eliminate either cyanide or azide reduction by V-nitrogenase. CO did, however, eliminate the inhibition of both electron flux and MgATP hydrolysis by CN^-, but not that caused by azide. These different responses to CO suggest different sites or modes of interaction for these two substrates with V-nitrogenase.

Azotobacter vinelandii is a strictly aerobic N_2-fixing organism that harbors three related, but genetically distinct, nitrogenases. Which one of these enzymes is operational at any particular time depends on which metal ions are present (*1*). For more than 50 years, the requirement of molybdenum for nitrogenase was thought to be mandatory (*2*), even though vanadium was demonstrated, early in the course of its study, to be almost as stimulatory as Mo with respect to the growth of some bacteria on N_2 as the sole nitrogen source (*3*). Despite these and other early experiments, the essentiality of Mo became entrenched. However, it is now clear that Mo is not essential for biological nitrogen fixation. Progress through the 1980s clearly identified Mo-independent nitrogenases (*4*), and the isolation of a V-containing nitrogenase (V-nitrogenase) from *Azotobacter chroococcum* established that one of the alternative systems was V-based (*5*), as predicted by the early studies. The use of either specific DNA probes or the characteristic ethane production from acetylene reduction has established the distribution of the Mo-independent nitrogenases among bacterial species (*1*, *6*, *7*). Although all N_2-fixing species studied so far have the Mo-containing nitrogenase (Mo-nitrogenase), it is still unclear why some species have only this single nitrogenase, whereas others have either two or three.

Like Mo-nitrogenase, the V- and Fe-nitrogenases comprise two separately purifiable component proteins. Each of these alternative nitrogenases has a specific homodimeric Fe protein[1] (herein called VnfH and AnfH, respectively) of ~60 kDa with a single [4Fe-4S] cluster. The second component

† Support from the National Institutes of Health (Grant DK 37255 to W.E.N.) is gratefully acknowledged.

* To whom correspondence should be addressed. Telephone: (540) 231-8431. Fax: (540) 231-9070. E-mail: wenewton@vt.edu.

‡ Permanent address: Center for Rhizobium Studies, School of Biological Sciences and Biotechnology, Division of Science, Murdoch University, Murdoch, Western Australia 6150, Australia.

[1] Abbreviations: ABTS, 2,2′-azino-di(3-ethyl)benthiazoline-6-sulfonic acid; Na_2-EDTA, disodium salt of ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FeMo cofactor, 7Fe-Mo-9S-homocitrate-X-containing prosthetic group in the MoFe protein; Fe protein (herein called NifH, VnfH, and AnfH), iron−protein component of Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase, respectively; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MgATP, magnesium salt of adenosine triphosphate; MoFe protein, VFe protein, and FeFe protein, molybdenum−iron, vanadium−iron, and iron−iron−protein components of Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase, respectively; P-cluster, 8Fe-7S-containing prosthetic group in the MoFe protein.

10.1021/bi0514109 CCC: $33.50
Published on Web 03/09/2006

(the VFe protein or FeFe protein) is ~200 kDa in mass but is an $\alpha_2\beta_2\gamma_2$ hexamer rather than an $\alpha_2\beta_2$ tetramer as found for the MoFe protein. These larger component proteins likely contain both types of prosthetic groups (the cofactor and P-clusters) found in the MoFe protein (*8*−*10*); however, X-ray crystallographic structures are available for only the Mo-nitrogenase and include those of the Fe protein (herein called NifH), the MoFe protein, and 2:1 Fe protein−MoFe protein complexes (*11*, *12*).

All three nitrogenases have the same requirements for catalytic activity, namely, MgATP, a low-potential reductant (usually sodium dithionite in vitro), and anaerobiosis. During the catalytic cycle, the two component proteins combine to form a complex, an electron is passed from the Fe protein (NifH, VnfH, or AnfH) to its partner (MoFe, VFe, or FeFe protein, respectively), and then the complex dissociates with the hydrolysis of MgATP. The relationship between electron transfer and MgATP hydrolysis is often quantified as the ATP:2e^- ratio, which under ideal conditions with Mo-nitrogenase is 4:1. Dissociation of the protein−protein complex is necessary to re-reduce the oxidized Fe protein and recharge it with MgATP, thereby making it competent to participate in another electron-transfer cycle (*13*). Because no substrate is reduced to product by a single electron, even the simplest substrate requires two such cycles. Furthermore, the component proteins of V-nitrogenase are known to form catalytically competent heterologous enzymes with the complementary proteins of Mo-nitrogenase, indicating a broad similarity in mechanism (*2*, *6*, *7*).

In addition to N_2, nitrogenases can reduce a number of other small molecules or ions, including acetylene and protons. However, both substrate reduction and inhibitor effects are clearly affected by the substitution of Mo with V. For example, (i) the efficiency of N_2 reduction decreases for V-nitrogenase and accounts for only ~50% of total electron flux under 101 kPa of N_2 compared to ~75% for Mo-nitrogenase (*14*); (ii) the preference for C_2H_2 reduction over H^+ reduction is much weaker for V-nitrogenase than for Mo-nitrogenase; (iii) during N_2 reduction only, the amount of MgATP hydrolyzed by V-nitrogenase for each electron pair appearing as product increases by 50% (*15*); (iv) no specific EPR signals are induced by CO during V-nitrogenase turnover (*16*); and (v) V-nitrogenase releases significant amounts of four-electron-reduced products, i.e., N_2H_4 during N_2 reduction (*14*) and C_2H_6 during C_2H_2 reduction (*15*), whereas wild-type Mo-nitrogenase releases neither.

Surprisingly, although both cyanide and azide have long held prominent positions among the long list of Mo-nitrogenase substrates (*17*, *18*), neither substrate has previously been tested with V-nitrogenase. The products of cyanide (HCN) reduction by Mo-nitrogenase are methane-plus-ammonia (a six-electron process) and methylamine (a four-electron process). Only HCN is a substrate; the CN^- also present in NaCN solutions is, in contrast, a potent inhibitor of electron flux through the enzyme. HCN reduction has been suggested to occur as shown in Scheme 1 (*19*, *20*).

As Scheme 1 shows, the catalyzed production of both CH_3NH_2 and, under some conditions, excess NH_3, i.e., an amount of NH_3 in excess of the amount of CH_4 produced, can be attributed to escape of initially formed intermediates from the active site. The four-electron-reduced intermediate

Scheme 1: HCN Reduction Catalyzed by Mo-Nitrogenase Likely Occurs by a Series of Two-Electron/Two-Proton Processes (where brackets indicate either substrate or intermediate bound to the enzyme)

$$\begin{array}{c} \overset{+2e^-/2H^+}{} \quad \overset{+2e^-/2H^+}{} \quad \overset{+2e^-/2H^+}{} \\ [HCN] \rightarrow [CH_2{=}NH] \rightarrow [CH_3NH_2] \rightarrow CH_4 + NH_3 \\ \downarrow \qquad\quad \downarrow \\ CH_2{=}NH \qquad CH_3NH_2 \\ \downarrow +H_2O \\ HCHO + NH_3 \end{array}$$

Scheme 2: Azide (both HN_3 and N_3^-) Reduction Catalyzed by Mo-Nitrogenase Occurs by a Six-Electron/Six-Proton and a Two-Electron/Two-Proton Process, Respectively, To Give N_2, N_2H_4, and NH_3[a]

$$HN_3 + 6e^- + 6H^+ \rightarrow N_2H_4 + NH_3 \qquad [1]$$

$$\begin{array}{l} N_3^- + 2e^- + 3H^+ \rightarrow N_2 + NH_3 \\ \qquad\qquad\qquad\quad \downarrow + 6e^-/6H^+ \\ \qquad\qquad\qquad\quad 2NH_3 \end{array} \qquad [2]$$

$$N_3^- + 8e^- + 9H^+ \rightarrow 3NH_3 \qquad [3]$$

[a] Excess NH_3 (see the text) has been suggested to occur either indirectly by reduction of N_2 formed in reaction 2 or directly by reaction 3.

on the reduction pathway to CH_4-plus-NH_3 is methylamine, which is stable toward hydrolysis and would be detectable. However, if the two-electron-reduced intermediate, methyleneimine ($CH_2{=}NH$), escapes the active site, it would be hydrolyzed to HCHO-plus-NH_3 and so could be the source of the excess NH_3. Formaldehyde has never been detected as a product of HCN reduction possibly because (i) both dithionite (*21*) and cyanide (*22*) interact with formaldehyde and so interfere with the Nash assay (*23*), (ii) insufficient excess NH_3 is produced by Mo-nitrogenase [although in contrast to our earlier results (*20*), Li et al. (*19*) showed significant excess NH_3 production], and (iii) formaldehyde may suffer further two-electron reduction to methanol. We, therefore, turned to V-nitrogenase to probe the reduction of HCN and the likely production of HCHO because of its propensity to release partially reduced intermediates, e.g., N_2H_4 during N_2 reduction. To do so, we developed and employed sensitive assay procedures for monitoring HCN reduction products in an attempt to identify either the released two-electron-reduced intermediate or the products of its hydrolysis.

A similar situation arises with catalyzed azide reduction (*24*−*26*; see Scheme 2, reactions 1−3). Here, excess NH_3, an amount greater than the combined amounts of N_2 and N_2H_4 produced, is also usually observed. At moderate concentrations (ca. 10 mM), azide is a good substrate for Mo-nitrogenase, and like the case for HCN reduction, it is reduced to give multiple products. However, unlike the case for catalyzed cyanide reduction, both HN_3 and N_3^- are substrates. Azide ion (N_3^-) is reduced by two electrons to give N_2-plus-NH_3, whereas hydrazoic acid (HN_3) is reduced by six electrons to N_2H_4-plus-NH_3. A major question is whether the excess NH_3 is a product of further catalyzed reduction of the N_2 produced (see Scheme 2, reaction 2) or a product of direct reduction of azide to NH_3 (see Scheme

2, reaction 3). We have also used V-nitrogenase to gain insight into this question.

EXPERIMENTAL PROCEDURES

Cell Growth and Protein Purification. The growth of a CA11.71 wild-type *A. vinelandii* strain containing the V-nitrogenase, nitrogenase derepression, and cell-extract preparation were performed as previously described (*20*) with the following exceptions. Ultrapure grade chemicals were utilized; contaminating Mo was removed from the sugar and phosphate before addition to the media (*27*, *28*), and 1 μM V_2O_5 was added to the media in place of Na_2MoO_4. Protein purification was exactly as described by Kim et al. (*29*). The purified VFe protein had a specific activity of 1000 nmol of H_2 min^{-1} (mg of protein)$^{-1}$, contained 1.1 mol of V/mol of protein, and had a 15:1 Fe:V ratio as determined by inductively coupled plasma emission spectroscopy. Because the Fe:V ratio is as expected for the holo-VFe protein but only approximately half of the expected V is present, this preparation is likely contaminated with an equal amount of a non-Fe-containing protein. No Mo (<0.01 mol of Mo/mol of protein) was detected in this preparation. The V:Fe ratio also indicates that there was no loss of metal-containing subunits during purification as previously reported (*30*). A limited amount of VnfH [specific activity of 1000 nmol of H_2 min^{-1} (mg of protein)$^{-1}$] was obtained from these preparations so that all data presented here use NifH [specific activity of 2200 nmol of H_2 min^{-1} (mg of protein)$^{-1}$] as the electron donor to the VFe protein. Some of the experiments were also performed using VnfH and produced identical results.

Nitrogenase Assays. Nitrogenase assays were assembled under argon in 9.5 mL glass serum vials with a butyl rubber stopper held on by an aluminum cap. The reaction mixture (1.0 mL final volume) contained 30 μmol of creatine phosphate, 25 μmol of HEPES buffer (pH 7.4), 20 μmol of sodium dithionite, 5 μmol of $MgCl_2$, 2.5 μmol of ATP, and 0.125 mg of creatine phosphokinase. An anaerobic NaCN stock solution was prepared by flushing solid NaCN in a sealed serum vial prior to the addition of degassed 25 mM HEPES (pH 7.4) and a predetermined amount of degassed 12 M HCl. This solution was added by syringe to each anaerobic assay vial at the start of the temperature preincubation period. Assays (ca. 0.25 mg of total protein in 1 mL unless otherwise stated) were initiated by addition via syringe of either VFe protein, followed immediately by Fe protein (either NifH mostly or VnfH), or a premixed 1:20 combination of the two. Unless otherwise stated, assays were terminated after 30 min by injection of 0.25 mL of 0.5 M Na_2-EDTA (pH 7.5). The time course assays contained 5 times as much of both proteins in a total volume of 1.5 mL. When required, CO was added by gastight Hamilton syringe (Hamilton Syringe Co., Reno, NV) to the appropriate assay vial during the preincubation period.

To determine the effect of H_2 on N_2 formation from N_3^- and its subsequent possible reduction to NH_3, 1.5 mL assays containing 10 mM sodium azide, 0.036 mg of VFe protein, and a 20-fold molar excess of NifH were run for 40 min in either the presence or absence of 101 kPa of H_2. Assay vials were initially degassed and filled with argon before the argon was replaced with H_2. Ammonia and hydrazine were assessed as described below.

The effect of pH on azide reduction was monitored in assays that contained either 1 or 5 mM azide. By varying the pH over the range of 7.15−6.2, we produced HN_3 concentrations varying from 2.8 to 24.5 μM and from 14 to 122 μM for the 1 and 5 mM azide concentrations, respectively.

Analytical Assays. (1) H_2 production was quantified gas chromatographically with a molecular sieve 5A column and a thermal conductivity detector. CH_4 was quantified with a Porapak N column and a flame ionization detector with He as the carrier gas.

(2) The measurement of MgATP hydrolysis from assays containing a creatine phosphokinase ATP-regenerating system was performed using the colorimetric method of Ennor (*31*), after pretreatment of samples as described by Dilworth et al. (*32*).

(3) NH_3 production was assessed from the same samples by the indophenol method (*33*) with the pretreatment of samples as described by Dilworth and Fisher (*34*).

(4) The amount of CH_3NH_2 was determined from 4 mL assays performed in 37 mL serum vials, containing 2 mg of total protein at a 20:1 Fe protein:VFe protein ratio, exactly as previously described (*20*).

(5) N_2H_4 production was assessed by adding a 0.8 mL aliquot of an assay to 1.2 mL of a *p*-dimethylaminobenzaldehyde solution and measuring the resulting absorbance at 458 nm (*24*).

(6) N_2 production from fully labeled potassium azide was assessed mass spectrometrically on a model 7070 E-HF mass spectrometer (VG analytical, Manchester, U.K.). The amount of N_2 produced was calculated from the $^{30}N_2$ peak compared to the internal Ar peak.

(7) Low levels of formaldehyde were measured colorimetrically with acetylacetone/ammonium acetate as described previously (*23*) in V-nitrogenase assays that contained 1.25 mg of total protein at a 20:1 Fe protein:VFe protein molar ratio and were assembled under Ar in 9.5 mL glass serum vials with a butyl rubber stopper held on by an aluminum cap. Spiked 1.5 mL assays, containing various HCHO concentrations from 0 to 100 nmol, received 0.2 mL of 1 M HCl before the proteins were added to prevent HCN reduction. Fully active assays were terminated after the appropriate incubation period at 30 °C by the addition of 0.2 mL of 1 M HCl. After analysis of the CH_4 that was produced, the rubber seals were removed and 30 μL of 1% starch was added. Assay vials were weighed prior to being titrated with 0.2 N I_2 to the starch end point (to oxidize the remaining dithionite). After the samples were reweighed to determine the new volume, 40 μL of 2 M $AgNO_3$ was added (to remove residual cyanide) and the total assay volume was centrifuged for 5 min at 14 000 rpm in a microfuge. Aliquots (1.9 mL) were combined with 30 μL of 7.5 M HCl and 0.5 mL of a 4× Nash reagent solution, which contained 0.8 mL of acetylacetone, 1.2 mL of glacial acetic acid, and 60 g of ammonium acetate in 100 mL, prior to incubation at 38 °C for 90 min. Samples were centrifuged again, and the A_{414} was determined in a 4 cm cell.

(8) Attempts to assess methanol production employed an enzymatic assay consisting of alcohol oxidase and peroxidase coupled to the oxidation of the chromogen 2,2′-azino-di(3-ethyl)benthiazoline-6-sulfonic acid (ABTS) (*35*). The MgATP to be used in these assays was placed under constant vacuum overnight to remove contaminating ethanol before use.

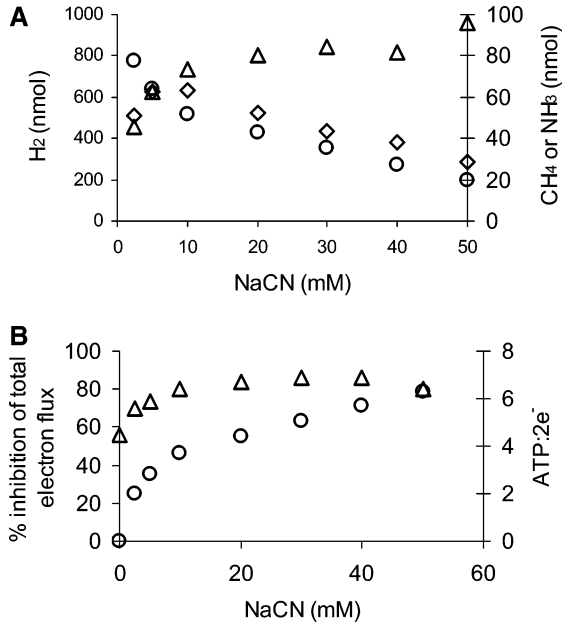

FIGURE 1: (A) Effect of NaCN concentration on H_2 (○), CH_4 (◇), and NH_3 (△) formation by V-nitrogenase. Assays were performed under 101 kPa of Ar, and each assay contained 0.039 mg of VFe protein and 0.21 mg of Fe protein (NifH). Assays were terminated after 12 min. (B) Inhibition of total electron flux [calculated as $2e^-$ for H_2, $6e^-$ for CH_4, and $2e^-$ for excess NH_3 (○)] and the resulting ATP:$2e^-$ ratio (△) as a function of the increase in NaCN concentration.

EDTA-quenched assays that either contained a known amount of methanol (0−50 nmol) or had turned over in the presence of sodium cyanide were passed through 6 cm Dowex AG1-X2 (Cl^-) anion-exchange Pasteur-pipet columns and then washed thoroughly with distilled water (3 × 0.5 mL). A 2 mL aliquot of the eluate was applied to a 2.5 cm Dowex-50X8 (H^+) Pasteur-pipet column and washed thoroughly with distilled water (2 × 0.5 mL). NaOH (1 M, 0.2 mL) was then added to each sample. Each aliquot (1.5 mL) was prepared for assay by addition of O_2-saturated ABTS/phosphate reagent (1 mL) to give a final assay concentration of 100 mM phosphate (pH 7.5) and 2.5 mg of ABTS. The assay was initiated by addition of 5 units of peroxidase and 0.01 unit of alcohol oxidase and the A_{414} determined after incubation for both 30 and 60 min at room temperature.

RESULTS

Extent of Product Formation as a Function of NaCN Concentration. The extent of formation of CH_4, H_2, and NH_3 as a function of NaCN concentration is presented in Figure 1 (top). Methylamine was not determined from these assays, and no adjustment for this omission has been made. The amount of H_2 produced decreases with an increase in NaCN concentration, and the amount of CH_4 formed increases initially but then declines at NaCN concentrations greater than 5 mM in parallel with H_2 production. In contrast, NH_3 production increases with an increase in NaCN concentration up to 20 mM NaCN but remains constant from 20 to 50 mM NaCN. These differing responses to NaCN concentration result in ca. 70 nmol of excess NH_3 being produced at the higher NaCN concentrations. Figure 1 (bottom) shows the corresponding inhibition of electron flux and the ATP:$2e^-$ ratio determined from the data presented in Figure 1 (top) (with no accounting for electrons used for methylamine production). These data also show that HCN reduction by V-nitrogenase is much less sensitive to cyanide inhibition (presumably by CN^-) than reduction by Mo-nitrogenase. Further, unlike that of Mo-nitrogenase, this inhibition of activity is accompanied by a similar decrease in the rate of ATP hydrolysis, which allows electron transfer to remain coupled to ATP hydrolysis (*20*).

In a separate experiment, the K_m value for CH_4 production from V-nitrogenase-catalyzed NaCN reduction over the range of 1−3.3 mM was calculated to be 1.9 mM. This value corresponds closely to the published value of 1.6 mM for the Mo-nitrogenase (*20*).

Determination of the Amount of Methylamine. The complexity of the method used to assess methylamine formation (*20*) made it impossible to assess product formation at all NaCN concentrations shown in Figure 1 (top). We, therefore, concentrated on assays containing either 5 or 50 mM NaCN because they produce either none or significant amounts of excess NH_3, respectively. Although in the presence of 5 mM NaCN no excess NH_3 is produced, CH_3NH_2 is clearly detectable. In fact, the CH_3NH_2:CH_4 ratio is 0.66:1, which is much higher than the published value of 0.35−0.39:1 for the Mo-nitrogenase under similar conditions (*19*, *20*). The rate of CH_3NH_2 production increases significantly at the higher NaCN concentration to become similar to that for methane production (which decreases), resulting in a CH_3NH_2:CH_4 ratio of ca. 1:1. Together with this increased rate of production of CH_3NH_2, there is also an increase in the rate of formation of excess NH_3 (see Table 1).

Recovery of HCHO from Steady-State Assays. Before we attempted to determine if HCHO was a product of HCN reduction, it was important to ensure that HCHO could be recovered and quantified when added to a typical nitrogenase assay. To do so, known amounts of HCHO were added to (i) assays turning over under 101 kPa of argon in the absence of HCN and (ii) assays that contained HCN and nitrogenase proteins but which were acidified with HCl so that no catalysis occurred. Figure 2 shows a typical HCHO recovery curve developed as described in Experimental Procedures for pre-acidified ("killed") assays containing 50 mM NaCN. When assays were allowed to turn over under an argon atmosphere in the absence of NaCN, the results were essentially identical (data not shown). Recovery was ≥80%.

Time Course of Formation of HCHO from Reduction of HCN by V-Nitrogenase. Figure 3 shows the production of HCHO, excess NH_3, and CH_4 as a function of time from nitrogenase assays containing 50 mM NaCN. The rates of formation of all three products were effectively linear for the first 15 min, but thereafter, all rates decreased until, by 30 min, product formation had effectively ceased. This loss of linearity likely reflects a MgATP and/or dithionite limitation because these assays contained 5 times as much protein but only 1.5 times as much MgATP and dithionite as normal assays. Ammonia and CH_4 production were assessed from the same assay vials, which were quenched by the addition of Na_2-EDTA, and showed that up to 100 nmol of excess NH_3 had been produced at assay times of ≥15 min. HCHO production was assessed using duplicate assays, which were quenched with HCl and showed that ca. 50 nmol of HCHO had been produced over the same time period.

Table 1: Product Formation, CH_3NH_2:CH_4 Ratio, and ATP:2e$^-$ Ratio from 5 and 50 mM NaCN by V-Nitrogenase and 5 mM NaCN by Mo-Nitrogenase[a]

		specific activity [nmol of electron pairs min^{-1} (mg of protein)$^{-1}$]					
N_2ase[b]	[NaCN] (mM)	H_2	CH_4	NH_3	CH_3NH_2	CH_3NH_2:CH_4 ratio	ATP:2e$^-$ ratio
V	0	980 ± 30	0	0	0	–	4.9
V	5	560 ± 9	59 ± 1.5	59 ± 4.5	39 ± 0.3	0.66:1	5.9
Mo[c]	5	270	150	150	58	0.39:1	20
V	50	256 ± 21	50 ± 1.5	65 ± 0.5	54 ± 6	1.1:1	6.8

[a] Nitrogenase assays were conducted in 4 mL reaction volumes under 101 kPa of Ar with a 20-fold molar excess of Fe protein (NifH). [b] N_2ase is nitrogenase. [c] Mo-nitrogenase data taken from ref *20*.

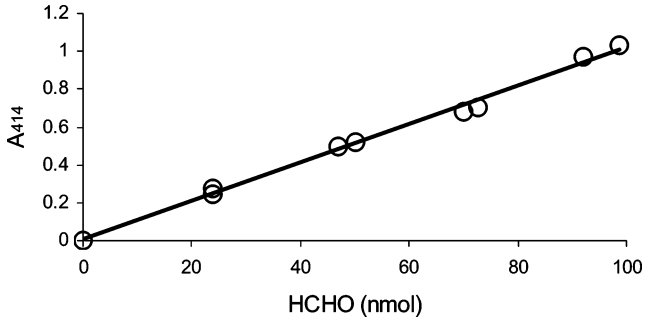

FIGURE 2: HCHO recovery curve for HCHO added to "prekilled" nitrogenase assays containing 50 mM NaCN and 1.25 mg of total protein at a 20:1 NifH:VFe protein molar ratio. Aliquots from vials containing a known amount of HCHO were assayed as described in Experimental Procedures.

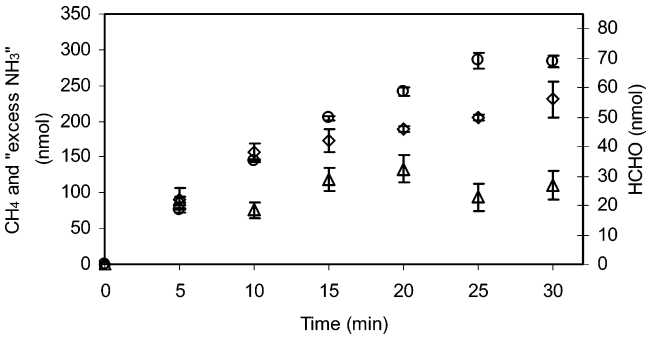

FIGURE 3: Time course for the production of CH_4 (O), HCHO (◇), and excess NH_3 (△) by V-nitrogenase. Assays were identical to those in Figure 2, except no HCHO was added and assays were allowed to run for 5−30 min. The total amount of NH_3 formed was determined from assays that were quenched by the addition of 0.35 mL of 0.5 M Na_2-EDTA, but only excess NH_3 production is plotted.

Attempts To Detect Methanol as a Product of HCN Reduction. Methanol (10−30 nmol) added to quenched nitrogenase assays was recovered in 75−80% yield after a 60 min incubation at room temperature with the ABTS/phosphate reagent. But "live" assays, which contained 50 mM NaCN and no added methanol, showed no increase in absorbance at 414 nm when compared to quenched control samples after being allowed to run for 40 min. Therefore, although these assays produced ca. 100 nmol of excess NH_3, they produced no methanol.

Reduction of Azide by VFe Protein. The apparent K_m for azide was determined at pH 7.4 by measuring the rate of N_2H_4 formation as a function of potassium azide concentration (0.8−20 mM). The value obtained was 2.6 mM (ca. 4 μM HN_3; data not shown), which is similar to the HN_3 value of 12 μM previously determined for the Mo-nitrogenase (*25*).

Table 2: Product Formation and ATP:2e$^-$ Ratio for Azide Reduction by V-Nitrogenase and Mo-Nitrogenase[a]

	specific activity [nmol of electron pairs min^{-1} (mg of protein)$^{-1}$]				
N_2ase[b]	N_2	N_2H_4	NH_3	H_2	ATP:2e$^-$ ratio
V	51 ± 3	53 ± 1	161 ± 3	470 ± 1	5.6
Mo[c]	630	160	880	1300	5.3

[a] Nitrogenase assays were conducted in 4 mL reaction volumes under 101 kPa of Ar with a 20-fold molar excess of Fe protein (NifH) in the presence of 10 mM $K^{15}N_3$. [b] N_2ase is nitrogenase. [c] Mo-nitrogenase data taken from ref *26*.

Table 2 shows that both N_2H_4 and N_2 were products of azide reduction with the V-nitrogenase. Assuming a reactivity similar to that of Mo-nitrogenase, then N_3^- is reduced to N_2 and HN_3 is reduced to N_2H_4, but at only 8 and 33%, respectively, of the rates with Mo-nitrogenase. Even so, both the VFe protein and the MoFe protein produce similar amounts of excess NH_3, i.e., NH_3 produced in excess of the sum of the amounts of N_2 and N_2H_4, but that produced by V-nitrogenase represents 8% of the total electron flux compared to only 3% for Mo-nitrogenase. Further, the VFe protein has equal rates of N_2 and N_2H_4 production, whereas the MoFe protein's rate of N_2 production is 4 times that for N_2H_4 formation. The amount of N_2 produced constitutes 7 and 21% of the total electron flux measured from the VFe and MoFe proteins, respectively, whereas the total amount of NH_3 formed is a similar percentage (22 and 30%, respectively) of the total electron flux for both enzymes. Unlike Mo-nitrogenase, V-nitrogenase suffers ca. 35% inhibition of both total electron flux and the rate of MgATP hydrolysis with 10 mM azide.

Is Azide or Hydrazoic Acid the Inhibitor of Electron Flux? The source of the inhibition of total electron flux induced by added azide was probed by monitoring H_2 evolution as a function of pH at either 1 or 5 mM sodium azide. Because HN_3 is a weak acid with a pK_a of 4.6 at 30 °C, varying the pH between 6 and 7 results in the calculated concentrations of HN_3 and N_3^- listed in Table 3, which also shows the resulting data for H_2 evolution. The trend is the same in both experiments and shows that the degree of inhibition of H_2 evolution increases with an increase in HN_3 concentration. The effect of HN_3 is more obvious with 1 mM azide, where the degree of inhibition increases by ca. 3-fold as the HN_3 concentration increases by ca. 9-fold. From these experiments, the rate of N_2H_4 production as a function HN_3 concentration gives a K_m value of 4 μM.

Is the N_2 Produced during N_3^- Reduction the Source of Excess NH_3? Because H_2 is a specific inhibitor of N_2 reduction, parallel experiments with V-nitrogenase were

Table 3: Effect of pH on HN_3 and N_3^- Concentrations and the Resulting Inhibition of H_2 Evolution by V-Nitrogenase[a]

	1 mM azide			5 mM azide		
pH	calcd [HN_3] (μM)	calcd [N_3^-] (μM)	% inhibition	calcd [HN_3] (μM)	calcd [N_3^-] (μM)	% inhibition
7.15	2.8	997	11	14	4986	36
6.78	6.6	994	17	33	4967	51
6.50	12.4	988	23	62	4938	50
6.20	24.5	976	32	122	4877	54

[a] Experiments were conducted as described in Experimental Procedures.

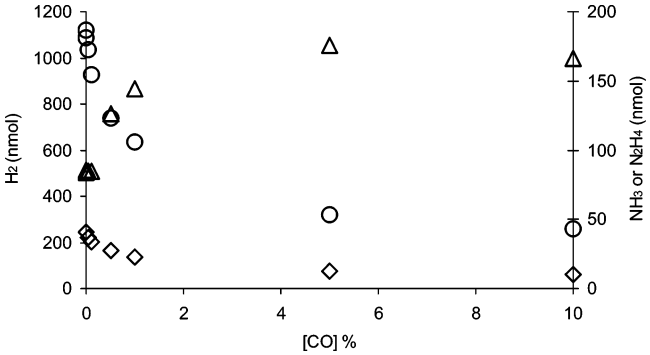

FIGURE 4: Effect of CO concentration on azide reduction to NH_3 (○) and N_2H_4 (◇), plus concomitant H_2 evolution (△), by V-nitrogenase. Assays were performed under 101 kPa of Ar, and the 1.5 mL reaction mixture contained 10 mM NaN_3 and 0.039 mg of VFe protein at a 20:1 NifH:VFe molar ratio. Appropriate amounts of CO were added with a gastight Hamilton syringe to give the desired final concentration. Assays were terminated after 45 min by the addition of 0.4 mL of 0.5 M Na_2-EDTA.

conducted with 10 mM sodium azide under either 101 kPa of Ar or 101 kPa of H_2. No significant difference was found in either N_2H_4 or NH_3 production. Assays conducted under H_2 yielded 169 ± 2 nmol of NH_3 and 37 ± 0.2 nmol of N_2H_4, whereas identical assays performed under an argon atmosphere yielded 161 ± 3 nmol of NH_3 and 37 ± 1 nmol of N_2H_4.

Effect of CO Concentration on Sodium Azide and Cyanide Reduction. Figure 4 shows the effect of CO on VFe protein-catalyzed azide reduction. As the CO concentration is increased incrementally to 10%, the amounts of both NH_3 and N_2H_4 decrease, but their loss is compensated by increases in the amount of H_2 that is evolved. However, unlike for Mo-nitrogenase, both NH_3 and N_2H_4 are still produced (at ca. 25% of their amounts under 101 kPa of Ar) in the presence of 10% CO. Furthermore, this CO-induced redistribution of electron flux does not relieve the inhibition of total electron flux caused by 10 mM NaN_3, which remained at 28% under 10% CO. There is also no significant change in the ATP:2e^- ratio at high CO concentrations, indicating that electron transfer remains coupled to ATP hydrolysis.

Figure 5 shows that CO has an effect similar to that observed during azide reduction when added to assays containing 10 mM NaCN. Again, the highest CO concentration tested (10%) is unable to completely inhibit HCN reduction. Approximately 20 and 15% of the amounts of NH_3 and CH_4, respectively, formed in the absence of CO are produced under 10% CO. Electron transfer remains coupled to ATP hydrolysis in both the presence and absence of CO.

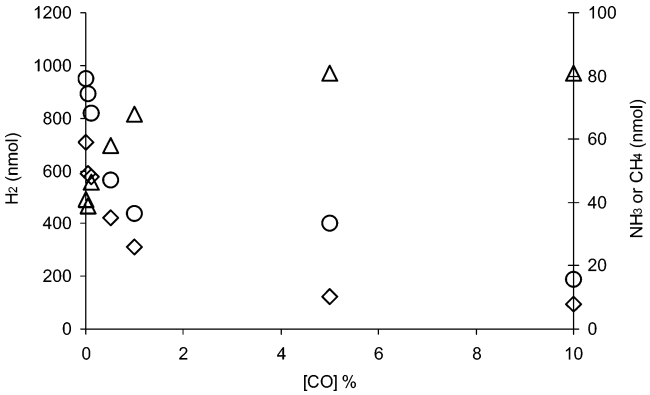

FIGURE 5: Effect of CO concentration on HCN reduction to NH_3 (○) and CH_4 (◇), plus concomitant H_2 evolution (△), by V-nitrogenase. Standard 1 mL nitrogenase assays were conducted under 101 kPa of Ar and contained 10 mM NaCN and 0.039 mg of VFe protein at a 20:1 NifH:VFe molar ratio. Appropriate amounts of CO were added with a gastight Hamilton syringe to give the desired final concentration. Assays were terminated after 30 min by the addition of 0.25 mL of 0.5 M Na_2-EDTA.

However, CO relieves the 30% "dead-end" inhibition of total electron flux caused by 10 mM NaCN.

DISCUSSION

The role of the heterometal (Mo or V) in nitrogenase function and, in particular, its potential to either bind substrates or influence their reduction continues to be debated. Although a substantial amount of evidence is accumulating that invokes the central iron atoms at the waist of the FeMo cofactor of the MoFe protein as a binding site for alkynes and alkenes (*36*−*42*), the binding site of other substrates, in particular, the most important substrate, N_2, remains uncertain. Replacing Mo with V gives rise to an enzyme still capable of catalyzing all the typical nitrogenase reactions, albeit usually in a less efficient manner (see the introductory section). Heterometal replacement provides an alternative approach to investigating the mechanism of biological nitrogen fixation, as do studies of the reduction of alternative substrates. Although neither azide nor cyanide has been tested with V-nitrogenase, they were both expected to be substrates but likely less efficiently used than with Mo-nitrogenase.

Catalyzed HCN Reduction. In general terms, V-nitrogenase catalyzes the reduction of HCN quite effectively when compared to Mo-nitrogenase. Total electron flux through V-nitrogenase is inhibited by CN^- as found for Mo-nitrogenase; however, 50% inhibition requires ∼15 mM NaCN compared to only 5 mM NaCN for Mo-nitrogenase. Furthermore, unlike that of Mo-nitrogenase, the rate of MgATP hydrolysis by V-nitrogenase is also similarly inhibited, resulting in electron transfer and MgATP hydrolysis remaining tightly coupled. This dead-end inhibition by CN^- is similar to that seen with high concentrations of NaCl (*43*, *44*). These observations indicate that the "single-HCN/CN^- binding site" hypothesis (*45*), in which CN^- binds and acts as an inhibitor until it is protonated to give the substrate, HCN, is unlikely with V-nitrogenase.

This high sensitivity to CN^- shown by Mo-nitrogenase presented a significant problem in our attempts to detect HCHO produced from catalyzed HCN reduction. When lower NaCN concentrations (up to 5 mM) were used, very

little (or no) excess NH_3 was produced, indicating that very little (or no) HCHO would be present (see Scheme 1 and Table 1) and our assay detected none. If higher NaCN concentrations were used (10−20 mM), then CN^- inhibition of electron flux through Mo-nitrogenase became sufficiently severe that the compromised turnover resulted in only a small amount of excess NH_3 and putatively insufficient HCHO for detection. However, two important differences between Mo-nitrogenase and V-nitrogenase suggested that the latter might offer a better opportunity for detecting HCHO. First, at higher NaCN concentrations, the VFe protein produces significant amounts of excess NH_3, whereas the MoFe protein produces much less. Second, the VFe protein releases considerably more CH_3NH_2 than does the MoFe protein (as judged by the CH_3NH_2:CH_4 ratio; see Table 1), consistent with a "leakier" active site.

Previous attempts to measure HCHO added to Mo-nitrogenase assays in the presence of both NaCN and dithionite had failed and resulted in the suggestion that any HCHO formed might not survive long enough to be detected in an assay (*19*). Although the HCHO assay protocol that we developed showed that HCHO added to assays can be recovered, we too failed to detect HCHO as a product of Mo-nitrogenase-catalyzed reduction of HCN for the reasons stated above. After attempting to detect and measure methanol, the likely two-electron-reduced product of further HCHO reduction, also without success, we chose to exploit the abundance of excess NH_3 and the efficient turnover of V-nitrogenase at high NaCN concentrations and returned to HCHO as the likely product of the reaction that produces excess NH_3.

The HCHO assay (*23*) with which we started was fraught with problems for our purpose. We found that residual dithionite inhibited color development with the Nash reagent. However, even after I_2 oxidation of the dithionite (*20*), color development remained inconsistent. Only after also removing residual cyanide by adding $AgNO_3$ were we able to recover >80% of the HCHO added to assays.

Applying this protocol to assays of HCN reduction catalyzed by V-nitrogenase, we detected and quantified product HCHO. However, the amount of HCHO detected was significantly smaller than the amount of excess NH_3 produced in the same assay vial. This discrepancy could be due problems such as low sensitivity and/or quantification, inherent to the assay; to another component of the assay that prevents full color development; or to formation of another product that remains undetected. If it is the latter, it is unlikely to be HCHO's two-electron reduction product, CH_3OH, for which we searched unsuccessfully.

Catalyzed Azide Reduction. Our studies of V-nitrogenase-catalyzed NaN_3 reduction also produced some interesting insights. Although the rates of formation of both N_2H_4 and N_2 are considerably lower than with Mo-nitrogenase, the K_m for N_2H_4 formation is actually lower than that of Mo-nitrogenase, indicating a higher apparent affinity for HN_3. We have found similar seemingly anomalous situations before (*46*). V-Nitrogenase exhibits no preference for the two-electron reduction of N_3^- to N_2-plus-NH_3 over the six-electron reduction of HN_3 to N_2H_4-plus-NH_3, whereas Mo-nitrogenase shows a clear preference for the two-electron process. Furthermore, production of excess NH_3 by V-nitrogenase is significantly greater than for Mo-nitrogenase. All of these product formation preferences of V-nitrogenase likely reflect the fact that its apparent affinity for substrate is higher than that of Mo-nitrogenase.

Unlike the case for Mo-nitrogenase (see Table 2; *25*, *26*, *47*), added NaN_3 inhibits equally both electron flux and the rate of MgATP hydrolysis with V-nitrogenase, resulting in an unchanging ATP:2e^- ratio, another example of dead-end inhibition. This difference may again reflect the stronger interaction between all azide forms and V-nitrogenase. The source of this inhibition most likely resides with HN_3 rather than N_3^-.

The source of excess NH_3 production from catalyzed azide reduction was investigated using the well-documented specific inhibition of N_2 reduction by H_2 (*14*, *15*, *47*). Parallel azide assays, one set under argon and another under H_2, resulted in identical rates of product formation. These results indicate that excess NH_3 formation is not a result of the further catalyzed reduction of product N_2 because the level of excess NH_3 formation would be expected to decrease in the presence of H_2 and it did not. Thus, eq 3 rather than eq 2 in Scheme 2 represents the source of excess NH_3 for V-nitrogenase. Previously, we reached the same conclusion for both wild-type and some variant Mo-nitrogenases (*20*, *47*). However, there are data in the literature which indicate that added H_2 does inhibit excess NH_3 formation from azide with wild-type Mo-nitrogenase (*19*). If this were so, then the mechanisms of azide reduction by Mo-nitrogenase and V-nitrogenase would be quite different.

Effect of Carbon Monoxide on Substrate Reduction. CO is a potent inhibitor of all Mo-nitrogenase-catalyzed substrate reductions, except that of protons. The situation with V-nitrogenase is less well defined but apparently more complex (*15*, *30*, *36*, *48*−*50*). Both azide and cyanide reduction by Mo-nitrogenase are completely eliminated by 1 kPa of CO (*19*, *20*, *24*); however, even at 10 kPa of CO, substantial substrate reduction continues with V-nitrogenase. Under these conditions, CO completely relieves the dead-end inhibition by cyanide; the resulting increased level of H_2 evolution compensates for both increased electron flux and attenuated HCN reduction. Under all conditions, electron flux remains tightly coupled to MgATP hydrolysis.

The effects of added CO on cyanide reduction by V-nitrogenase do not fully carry over to azide reduction. One similarity is that substrate reduction continues at a diminished rate with added CO. However, unlike that with cyanide, this redistribution of electron flux does not relieve the dead-end inhibition phenomenon. Both azide-induced electron-flux inhibition and the rate of MgATP hydrolysis are unaffected by added CO, resulting in an unchanging ATP:2e^- ratio. These responses to CO are completely different from those of Mo-nitrogenase, where there is neither electron-flux inhibition nor MgATP-hydrolysis inhibition by azide at pH 7.4 and where as little as 1 kPa of CO completely eliminates all product formation from azide (*47*). These results are consistent with weaker binding of CO to V compared to the binding to Mo and are compatible with an earlier observation that CO is a less effective inhibitor of production of C_2H_4 from C_2H_2 with V-nitrogenase than with Mo-nitrogenase (*14*).

Although all available evidence indicates that the structures of the FeMo cofactor and FeV cofactor are similar, our data show that the presence of V obviously impacts catalyzed

substrate reduction. Would such an impact be expected if the heterometal has little (or no) involvement in the electronic structure of the cofactor? And, consequently, on catalysis? These questions remain open.

ACKNOWLEDGMENT

We thank Prof. Paul Bishop (North Carolina State University, Raleigh, NC) for the generous gift of *A. vinelandii* strain CA11.71.

REFERENCES

1. Luque, F., and Pau, R. N. (1991) Transcriptional regulation by metals of structural genes for *Azotobacter vinelandii* nitrogenases, *Mol. Gen. Genet. 227*, 481−487.
2. Newton, W. E. (1993) Nitrogenases: Distribution, composition, structure and function, in *New Horizons in Nitrogen Fixation* (Palacios, R., Mora, J., and Newton, W. E., Eds.) pp 5−18, Kluwer Academic, Dordrecht, The Netherlands.
3. Bortels, H. (1933) Short note on the catalysis of biological nitrogen fixation, *Zentralbl. Bakteriol. Parasitenkd. 87*, 476−477.
4. Bishop, P. E., Jarlenski, D. M. L., and Hetherington, D. R. (1980) Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*, *Proc. Natl. Acad. Sci. U.S.A. 77*, 7342−7346.
5. Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. E., and Postgate, J. R. (1986) The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme, *Nature 322*, 388−390.
6. Eady, R. R. (1996) Structure−function relationships of alternative nitrogenases, *Chem. Rev. 96*, 3013−3030.
7. Eady, R. R. (2003) Current status of structure function relationships of vanadium nitrogenase, *Coord. Chem. Rev. 237*, 23−30.
8. Smith, B. E., Eady, R. R., Lowe, D. J., and Gormal, C. (1988) The vanadium−iron protein of vanadium nitrogenase from *Azotobacter chroococcum* contains an iron−vanadium cofactor, *Biochem. J. 250*, 299−302.
9. Ravi, N., Moore, V., Lloyd, S. G., Hales, B. J., and Huynh, B. H. (1994) Mössbauer characterization of the metal clusters in *Azotobacter vinelandii* nitrogenase VFe protein, *J. Biol. Chem. 269*, 20920−20924.
10. Krahn, E., Weiss, B. J. R., Kröckel, M., Groppe, J., Henkel, G., Cramer, S. P., Trautwein, A. X., Schneider, K., and Müller, A. (2002) The Fe-only nitrogenase from *Rhodobacter capsulatus*: Identification of the cofactor, an unusual high-nuclearity iron−sulfur cluster, by Fe *K*-edge EXAFS and ^{57}Fe Mössbauer spectroscopy, *J. Biol. Inorg. Chem. 7*, 37−45.
11. Rees, D. C., and Howard, J. B. (2000) Nitrogenase: Standing at the crossroads, *Curr. Opin. Chem. Biol. 4*, 559−566.
12. Einsle, O., Tezcan, F. A., Andrade, S. L., Schmid, B., Yoshida, M., Howard, J. B., and Rees, D. C. (2002) Nitrogenase MoFe-protein at 1.16 Å resolution: A central ligand in the FeMo-cofactor, *Science 297*, 1696−1700.
13. Lowe, D. J., and Thorneley, R. N. F. (1984) The mechanism of *Klebsiella pneumoniae* nitrogenase action, *Biochem. J. 224*, 877−909.
14. Dilworth, M. J., and Eady, R. R. (1991) Hydrazine is a product of dinitrogen reduction by the vanadium−nitrogenase from *Azotobacter chroococcum*, *Biochem. J. 277*, 465−468.
15. Dilworth, M. J., Eady, R. R., and Eldridge, M. E. (1988) The vanadium nitrogenase of *Azotobacter chroococcum*, *Biochem. J. 249*, 745−751.
16. Maskos, Z., and Hales, B. J. (2002) Photo-lability of CO bound to Mo-nitrogenase from *Azotobacter vinelandii*, *J. Inorg. Biochem. 93*, 11−17.
17. Biggins, D. R., and Kelly, M. (1970) Interaction of nitrogenase from *Klebsiella pneumoniae* with ATP or cyanide, *Biochim. Biophys. Acta 205*, 288−299.
18. Hardy, R. W. F., and Knight, E., Jr. (1967) ATP-dependent reduction of azide and HCN by N_2-fixing enzymes of *Azotobacter vinelandii* and *Clostridium pasteurianum*, *Biochim. Biophys. Acta 139*, 69−90.
19. Li, J. G., Burgess, B. K., and Corbin, J. L. (1982) Nitrogenase reactivity: Cyanide as substrate and inhibitor, *Biochemistry 21*, 4393−4402.
20. Fisher, K., Dilworth, M. J., Kim, C. H., and Newton, W. E. (2000) *Azotobacter vinelandii* nitrogenases with substitutions in the FeMo-cofactor environment of the MoFe protein: Effects of acetylene or ethylene on interactions with H^+, HCN, and CN^-, *Biochemistry 39*, 10855−10865.
21. Wilson, C. L., and Wilson D. W. (1962) *Comprehensive Analytical Chemistry*, p 289, Elsevier, London.
22. von Richter, V. (1947) in *Richter's Organic Chemistry* (Anschütz, R., and Reindel, F., Eds.) 3rd ed. (English), Vol. 1, p 245, Elsevier, London.
23. Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction, *Biochem. J. 55*, 416−421.
24. Dilworth, M. J., and Thorneley, R. N. F. (1981) Nitrogenase of *Klebsiella pneumoniae*: Hydrazine is as product of azide reduction, *Biochem. J. 193*, 971−983.
25. Rubinson, J. F., Burgess, B. K., Corbin, J. L., and Dilworth, M. J. (1985) Nitrogenase reactivity: Azide reduction, *Biochemistry 24*, 273−283.
26. Fisher, K., Dilworth, M. J., and Newton, W. E. (2000) Differential effects on N_2 binding and reduction, HD formation, and azide reduction with α-195^{His} and α-191^{Gln}-substituted MoFe proteins of *Azotobacter vinelandii* nitrogenase, *Biochemistry 39*, 15570−15577.
27. Joerger, R. D., Wolfinger, E. D., and Bishop, P. E. (1991) The gene encoding dinitrogenase reductase-2 is required for expression of the second alternative nitrogenase from *Azotobacter vinelandii*, *J. Bacteriol. 173*, 4440−4446.
28. Premakumar, R., Loveless, T. M., and Bishop, P. E. (1994) Effect of amino acid substitutions in a potential metal-binding site of AnfA on expression from the *anfH* promoter in *Azotobacter vinelandii*, *J. Bacteriol. 176*, 6139−6142.
29. Kim, C. H., Newton, W. E., and Dean, D. R. (1995) Role of the MoFe protein α-subunit histidine-195 residue in FeMo-cofactor binding and nitrogenase catalysis, *Biochemistry 34*, 2798−2808.
30. Blanchard, C. Z., and Hales, B. J. (1996) Isolation of two forms of the nitrogenase VFe protein from *Azotobacter vinelandii*, *Biochemistry 35*, 472−478.
31. Ennor, A. H. (1957) Determination and preparation of N-phosphates of biological origin, *Methods Enzymol. 3*, 850−856.
32. Dilworth, M. J., Eldridge, M. E., and Eady, R. R. (1992) Correction for creatine interference with the direct indophenol measurement of NH_3 in steady-state nitrogenase assays, *Anal. Biochem. 207*, 6−10.
33. Fawcett, J. K., and Scott, J. E. (1960) Determination of ammonia using indophenol, *J. Clin. Pathol. 13*, 156−159.
34. Dilworth, M. J., and Fisher, K. (1998) Elimination of creatine interference with the indophenol measurement of NH_3 produced during nitrogenase assays, *Anal. Biochem. 256*, 242−244.
35. Herzberg, G. R., and Rogerson, M. (1985) Use of alcohol oxidase to measure the methanol produced during the hydrolysis of D- and L-methyl-3-hydroxybutyric acid, *Anal. Biochem. 149*, 354−357.
36. Benton, P. M., Laryukhin, M., Mayer, S. M., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2003) Localization of a substrate binding site on the FeMo-cofactor in nitrogenase: Trapping propargyl alcohol with an α-70-substituted MoFe protein, *Biochemistry 42*, 9102−9109.
37. Igarashi, R. Y., Dos Santos, P. C., Niehaus, W. G., Dance, I. G., Dean, D. R., and Seefeldt, L. C. (2004) Localization of a catalytic intermediate bound to the FeMo-cofactor of nitrogenase, *J. Biol. Chem. 279*, 34770−34775.
38. Lee, H. I., Igarashi, R. Y., Laryukhin, M., Doan, P. E., Dos Santos, P. C., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2004) An organometallic intermediate during alkyne reduction by nitrogenase, *J. Am. Chem. Soc. 126*, 9563−9569.
39. Davis, L. C., Henzl, M. T., Burris, R. H., and Orme-Johnson, W. H. (1979) Iron−sulfur clusters in the molybdenum−iron protein component of nitrogenase. Electron paramagnetic resonance of the carbon monoxide inhibited state, *Biochemistry 18*, 4860−4869.
40. Shen, J., Dean, D. R., and Newton, W. E. (1997) Evidence for multiple substrate-reduction sites and distinct inhibitor-binding sites from an altered *Azotobacter vinelandii* nitrogenase MoFe protein, *Biochemistry 36*, 4884−4894.
41. Christiansen, J., Seefeldt, L. C., and Dean, D. R. (2000) Competitive substrate and inhibitor interactions at the physiologically relevant active site of nitrogenase, *J. Biol. Chem. 275*, 36104−36107.
42. Han, J., and Newton, W. E. (2004) Differentiation of acetylene-reduction sites by stereoselective proton addition during *Azoto-*

bacter vinelandii nitrogenase-catalyzed C_2D_2 reduction, *Biochemistry 43*, 2947−2956.
43. Burns, A., Watt, G. D., and Wang, Z. C. (1985) Salt inhibition of nitrogenase catalysis and salt effects on the separate protein components, *Biochemistry 24*, 3932−3936.
44. Deits, T. L., and Howard, J. B. (1990) Effects of salts on *Azotobacter vinelandii* nitrogenase activities, *J. Biol. Chem. 265*, 3859−3867.
45. Lowe, D. J., Fisher, K., Thorneley, R. N. F., Vaughn, S. A., and Burgess, B. K. (1989) Kinetics and mechanism of the reaction of cyanide with molybdenum nitrogenase from *Azotobacter vinelandii*, *Biochemistry 28*, 8460−8466.
46. Maskos, Z., Fisher, K., Sørlie, M., Newton, W. E., and Hales, B. J. (2005) Variant MoFe proteins of *Azotobacter viinelandii*: Effects of carbon monoxide on electron paramagnetic resonance spectra generated during enzyme turnover, *J. Biol. Inorg. Chem. 10*, 394−406.
47. Dilworth, M. J., Fisher, K., Kim, C. H., and Newton, W. E. (1998) Effects on substrate reduction of substitution of histidine-195 by glutamine in the α-subunit of the *Azotobacter vinelandii* MoFe protein of *Azotobacter vinelandii* nitrogenase, *Biochemistry 37*, 17495−17505.
48. Cameron, L. M., and Hales, B. J. (1996) Unusual effect of CO on C_2H_2 reduction by V nitrogenase from *Azotobacter vinelandii*, *J. Am. Chem. Soc. 118*, 279−280.
49. Cameron, L. M., and Hales, B. J. (1998) Investigation of CO binding and release from Mo-nitrogenase during catalytic turnover, *Biochemistry 37*, 9449−9456.
50. Moore, V. G., Tittsworth, R. C., and Hales, B. J. (1994) Construction and characterization of hybrid component I from V-nitrogenase containing FeMo-cofactor, *J. Am. Chem. Soc. 116*, 12101−12102.

BI0514109