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^- †

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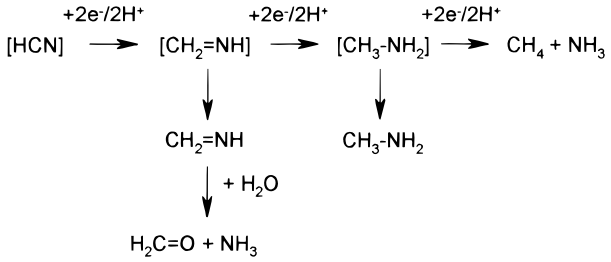
Received January 24, 2000; Revised Manuscript Received April 24, 2000

ABSTRACT: Wild-type and three altered *Azotobacter vinelandii* nitrogenase MoFe proteins, with substitutions either at α-195^{His} (replaced by α-195^{Asn} or α-195^{Gln}) or at α-191^{Gln} (replaced by α-191^{Lys}), were used to probe the interactions of HCN and CN^-, both of which are present in NaCN solutions at pH 7.4, with nitrogenase. The first goal was to determine how added C_2H_2 enhances the rate of CH_4 production from HCN reduction by wild-type nitrogenase. In the absence of C_2H_2, wild-type Mo-nitrogenase showed a declining total electron flux, which is an overall measure of all products formed, as the NaCN concentration was increased from 1 to 5 mM, whereas the rates of both CH_4 and NH_3 production increased with increasing NaCN concentration. The NH_3 production rate exceeded the CH_4 production rate up to 5 mM NaCN, at which point they became equal. The "excess NH_3" likely arises from the two-electron reduction of HCN to CH_2═NH, some of which is released and hydrolyzed to HCHO plus NH_3. With added C_2H_2, the rate of CH_4 production increased but only until it equaled that of NH_3 production, which remained unchanged. In addition, total electron flux was decreased even more at each NaCN concentration by C_2H_2. The increased CH_4 production did not arise from the added C_2H_2. The lowered total electron flux with C_2H_2 present would decrease the affinity of the enzyme for HCN, making it a poorer competitor for the binding site. Thus, less CH_2═NH would be displaced, more CH_2═NH would undergo the full six-electron reduction, and the rate of CH_4 production would be enhanced. A second goal was to gain mechanistic insight into the roles of the amino acid residues in the α-subunit of the MoFe protein at positions α-191 and α-195 in substrate reduction. At 5 mM NaCN and in the presence of excess wild-type Fe protein, the specific activity for CH_4 production by the α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins was 59%, 159%, and 6%, respectively, of that of wild type. For the α-195^{Asn} MoFe protein, total electron flux decreased with increasing NaCN concentration like wild type. However, the rates of both CH_4 and NH_3 production were maximal at 1 mM NaCN, and they remained unequal even at 5 mM NaCN. With the α-195^{Gln} MoFe protein, the rates of production of both CH_4 and NH_3 were equal at all NaCN concentrations, and total electron flux was hardly affected by changing the NaCN concentration. With the α-191^{Lys} MoFe protein, the rates of both CH_4 and NH_3 production were very low, but the rate of NH_3 production was higher, and both rates slowly increased with increasing NaCN concentration. A hypothesis, which is based on the varying apparent affinities of the altered MoFe proteins for HCN and CN^-, is advanced to explain the higher rate of NH_3 production versus the rate of CH_4 production and the effect of increasing NaCN concentration on electron flux to products. A new method for CH_3NH_2 quantification showed that all four MoFe proteins produced CH_3NH_2. Added CO significantly inhibited both CH_4 and NH_3 production from HCN with all MoFe proteins except for the α-191^{Lys} MoFe protein, which still manifested its very low rate of NH_3 production but without CH_4 production. All of the MoFe proteins responded differently to the addition of C_2H_2 to reactions containing NaCN. With the α-195^{Asn} MoFe protein, added C_2H_2 decreased the rates of both CH_4 and NH_3 production, but the rate of NH_3 production decreased much less. C_2H_2 also exacerbated the inhibition of electron flux. With the α-195^{Gln} MoFe protein, added C_2H_2 decreased the rates of both CH_4 and NH_3 production substantially and about equally. C_2H_2 also eliminated the slight decrease in total electron flux that was caused by NaCN. Added C_2H_2 hardly affected the α-191^{Lys} MoFe protein. The effects of adding C_2H_2 appear related to the different relative affinities of the various MoFe proteins for HCN versus C_2H_2. Added C_2H_4 had no effect on HCN reduction with any of the MoFe proteins. The data are consistent with the "single HCN/CN^- binding site" hypothesis [Lowe, D. J., Fisher, K., Thorneley, R. N. F., Vaughn, S. A., and Burgess, B. K. (1989) *Biochemistry 28*, 8460−8466], imply important roles for both residues, especially α-191^{Gln}, in the catalysis of HCN reduction, and suggest that different substrates may use different proton delivery routes.

Molybdenum nitrogenase (Mo-nitrogenase), one of three genetically distinct forms of the enzyme, catalyzes the conversion of N_2 into usable ammonia. It consists of two metalloproteins. The Fe protein,[1] a homodimer of about 65 kDa, has two MgATP binding sites and acts as the specific electron donor to the MoFe protein, a heterotetramer of about 230 kDa. The MoFe protein contains two each of two types of metal-containing prosthetic groups. The FeMo-cofactors carry the binding sites for small-molecule substrates (*1*−*3*), whereas the P clusters act as intermediates in the transfer of

10.1021/bi0001628 CCC: $19.00
Published on Web 08/09/2000

Scheme 1: Reduction Sequence for the Catalyzed Two-, Four-, and Six-Electron Reduction of HCN by Mo-Nitrogenase[a]



[a] [ ] indicates substrate bound to the enzyme.

electrons from the Fe protein through the MoFe protein to bound substrate (*4*−*7*). The interactions of the component proteins involve cycles of association and dissociation with each cycle accompanied by hydrolysis of a minimum of two molecules of MgATP for every electron transferred (*8*, *9*).

When given an anaerobic environment, a low-potential reductant, and a source of MgATP, Mo-nitrogenase catalyzes the reduction not only of N_2 but also of many other so-called "alternative" substrates, including proton reduction to H_2 (*10*), C_2H_2 reduction to C_2H_4 (*11*, *12*), and cyanide reduction to CH_4, NH_3, and CH_3NH_2 (*13*−*15*). Because HCN is a relatively weak acid ($pK_a = 9.11$), sodium cyanide solutions simultaneously contain both HCN and CN^-, the relative amounts varying with pH. The actual nitrogenase substrate is HCN. Under some conditions, its reduction by Mo-nitrogenase results in "excess NH_3", i.e., an amount of NH_3 in excess of the CH_4 produced (*15*). The occurrence of excess NH_3 and CH_3NH_2 (methylamine) is attributed to a series of two-electron/two-proton steps in a reduction pathway (Scheme 1) initially converting HCN into CH_2═NH (methyleneimine). When any of this intermediate escapes from the active site, it is hydrolyzed to H_2C═O (which has never been detected) and excess NH_3 (*15*). Additional two-electron/two-proton steps would yield CH_3NH_2, some of which also escapes, and finally CH_4 and NH_3.

Interestingly, addition of C_2H_2 to wild-type Mo-nitrogenase, when reducing HCN, enhances CH_4 production (*14*, *16*), implying that both HCN and C_2H_2 are bound simultaneously to separate sites on nitrogenase.

The anion, CN^-, acts not as a substrate of wild-type Mo-nitrogenase but as a potent inhibitor of electron flux from the Fe protein through the MoFe protein to substrate. Cyanide ion, however, does not inhibit MgATP hydrolysis. This situation results in an increase in the ATP/2e$^-$ ratio, which measures the number of MgATP molecules hydrolyzed for each pair of electrons appearing in products. On the basis of pre-steady-state data (*17*), the suggestion that CN^- and HCN bind at independent sites (*15*) has been questioned. The more recent interpretation suggests that both inhibitor and substrate bind at the same site and that CN^- only acts as an electron flux inhibitor until it is protonated to give the substrate HCN. This behavior contrasts with that of CO, which is a noncompetitive inhibitor of the catalyzed reduction of all substrates except H^+ (*18*, *19*). All electron flux is then diverted to H_2 evolution so that neither the rate of electron flux through the enzyme nor the rate of MgATP hydrolysis is affected, resulting in an unchanged ATP/2e$^-$ ratio. Added CO and, to some extent, azide can reverse the inhibition of electron flux caused by CN^- with wild-type Mo-nitrogenase (*15*).

The three-dimensional crystal structures of both component proteins of Mo-nitrogenase have been solved by X-ray techniques (*6*, *20*−*23*). These structures have defined the bonding of the FeMo-cofactor within the MoFe protein. We have sought insight into the spatial arrangement of the binding sites for the various nitrogenase substrates and inhibitors by disrupting selected localized areas of the FeMo-cofactor's environment through directed amino acid substitutions (*24*−*29*). Two major targets have been the α-subunit residues, α-195^{His} and α-191^{Gln}, neither of which is covalently bonded to the FeMo-cofactor. However, the ϵ-nitrogen of the imidazole ring of α-195^{His} approaches to within 3.2 Å (*30*) and forms a putative hydrogen bond to one member of the triangle of sulfides that form the "waist" of the FeMo-cofactor. The amide N of α-191^{Gln} hydrogen-bonds with a terminal carboxylate of (*R*)-homocitrate, which is ligated to the Mo atom of the FeMo-cofactor by its β-hydroxyl and β-carboxylate groups (Figure 1).

Substitution of the α-195^{His} residue by a number of other amino acids resulted in mutant strains that were unable to reduce N_2 at appreciable rates, although all retained activity for reduction of both H^+ and C_2H_2 (*3*, *26*, *31*). The MoFe protein in which the α-195^{His} was replaced with glutamine (abbreviated as α-195^{Gln} MoFe protein) resembles wild type in its interactions with the substrates, H^+ and acetylene, and with the inhibitor, CO. However, N_2, which is only very poorly reduced, behaves primarily as a reversible inhibitor of electron flux (*26*, *31*). The α-195^{Gln} MoFe protein is largely unaffected by CN^- (*31*), which in wild-type acts as a potent inhibitor of electron flux to substrate (*15*).

In contrast, substitutions at the α-191^{Gln} residue resulted in some strains that still fix N_2. Unlike the α-195^{His}-substituted MoFe proteins, all the α-191^{Gln}-substituted MoFe proteins exhibited H_2 evolution that was sensitive to the presence of CO and relatively ineffective C_2H_2 reduction. One similarity exists, however, because both the α-191^{Lys} and α-191^{Glu} MoFe proteins and the α-195^{Asn} MoFe protein form small amounts of C_2H_6 as a product of C_2H_2 reduction (*3*, *32*, *33*). No data are available concerning either HCN reduction or electron flux inhibition by CN^- with any α-191^{Gln}-substituted MoFe protein.

Here, we have used the wild-type, α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins to probe the interactions among a selection of substrates and inhibitors of nitrogenase. We had two major goals for this study. First, we wanted to determine if added C_2H_2 enhances the rate of NH_3 production as well

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

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[1] Abbreviations: MoFe protein, the larger molybdenum- and iron-containing protein of nitrogenase; Fe protein, the smaller protein component of nitrogenase that contains iron only; FeMo-cofactor, the molybdenum- and iron-containing prosthetic group of the MoFe protein; MgATP, the magnesium salt of adenosine triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA-Na_2, the disodium salt of ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

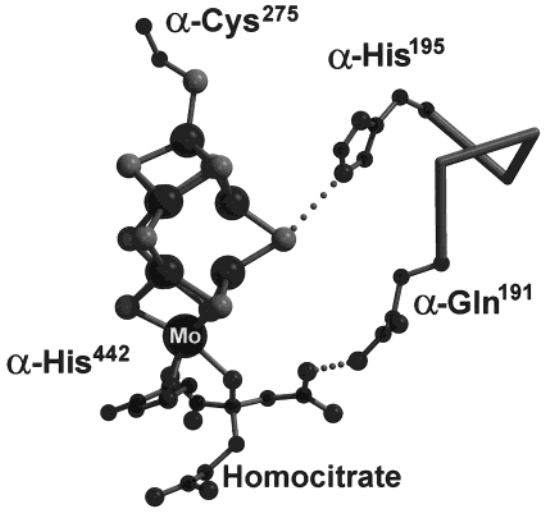

FIGURE 1: View of the FeMo-cofactor environment of the wild-type nitrogenase MoFe protein (*6*, *21*−*23*). The two directly bonded residues, α-275^{Cys} and α-442^{His}, are shown plus the two residues, α-191^{Gln} and α-195^{His}, that were substituted in this work. Homocitrate (at the bottom) provides two bonds to the Mo atom. The putative −NH→O− hydrogen bond between the amide function of α-191^{Gln} and homocitrate and the putative −NH→S− hydrogen bond between the ϵ-N of the imidazole ring of α-195^{His} and one of the three sulfides, which hold the two subclusters of the FeMo-cofactor together, are indicated by dotted lines. The Fe atoms of the FeMo-cofactor are the largest, black spheres, whereas the S atoms are the smaller, lighter-colored spheres. The C atoms are the smallest, black spheres, and both the O and N atoms are the intermediate-size, black spheres. The figure was drawn using MOLSCRIPT (*46*).

as that of CH_4 production from HCN reduction, whether added C_2H_4 would be similarly effective, and what the basis for this enhancement was. Previous studies (*14*, *16*) of this phenomenon were incomplete because the concentration of C_2H_2 used was not always stated and data concerning electron flux to products was not available because neither the rate of NH_3 production nor the rate of concomitant H_2 evolution was measured. Our ability to measure all products of an assay and the extension of these studies to the altered MoFe proteins were expected to give considerable insight into this phenomenon. Second, we sought to gain mechanistic insight into the functioning of Mo-nitrogenase through its interaction(s) with HCN and CN^-. In particular, we were interested in how and through what channels are electrons and protons delivered to substrate. We initiated this investigation by measuring the impact of selected amino acid substitutions on (i) the relative rates of product formation (CH_4, NH_3, CH_3NH_2, and H_2) during HCN reduction, (ii) the inhibition of electron flux by CN^-, and (iii) the effect of added C_2H_2, C_2H_4, or CO.

EXPERIMENTAL PROCEDURES

Cell Growth and Protein Purification. The growth of wild-type (α-191^{Gln}/α-195^{His}), DJ255 (α-191^{Lys}), DJ178 (α-195^{Asn}), and DJ540 (α-195^{Gln}) strains of *Azotobacter vinelandii*, nitrogenase derepression, and cell extract preparation were performed as previously described (*32*, *33*). The purification of the nitrogenase MoFe protein component involved (*26*) (i) Q-Sepharose anion-exchange chromatography using a linear NaCl concentration gradient, (ii) gel filtration on Sephacryl S-200, and (iii) phenyl-Sepharose hydrophobic interaction chromatography. After the first Q-Sepharose column, the Fe protein fraction was purified to homogeneity on a second Q-Sepharose anion-exchange column, resulting in a specific activity of about 2800 nmol of H_2 produced·(min·mg)$^{-1}$. The purified component proteins were concentrated, using an Amicon microfiltration cell in an ice−water bath and exchanged into 25 mM HEPES (pH 7.4) by passage through an anaerobic P-6DG column. The resulting specific activities are listed in Table 1. The method of Lowry et al. (*34*) was used for protein concentration determinations, and SDS−PAGE with Coomassie Blue staining was used to confirm that all proteins were homogeneous (*35*). All buffers were saturated with argon and contained 2 mM sodium dithionite. Metal content was measured by inductively coupled plasma atomic emission spectroscopy on a Perkin-Elmer Plasma 400 spectrometer. The molybdenum content varied among the four purified MoFe proteins but not the iron-to-molybdenum ratio, which was constant within the range of 13−14:1. Both the wild-type and α-195^{Gln} MoFe proteins contained 1.9 Mo atoms per molecule, whereas the α-195^{Asn} and α-191^{Lys} MoFe proteins contained 0.9 Mo atom per molecule. These values indicate that both the α-195^{Asn} and α-191^{Lys} MoFe protein preparations may be mixtures of approximately equal amounts of holo- and apo-MoFe protein.

Table 1: Product Formation, Total Electron Flux, and ATP/2e$^-$ Ratio for HCN Reduction Using MoFe Proteins from Wild-Type and Mutant Strains

MoFe protein	conditions[a]	electron pairs appearing in products[b] H_2	CH_4	NH_3	total SA[c]	ATP/2e$^-$ [d]
wild type	Ar alone	2900	0	0	2900	4.6
	+NaCN	510	270	260	1040	14
	+NaCN/CO	2900	9	22	2931	4.4
α-195^{Asn}	Ar alone	1400	0	0	1400	4.7
	+NaCN	360	160	200	720	9.0
	+NaCN/CO	1400	1	0	1401	5.0
α-195^{Gln}	Ar alone	2900	0	0	2900	4.6
	+NaCN	1700	430	420	2550	6.0
	+NaCN/CO	3200	0	0	3200	4.5
α-191^{Lys}	Ar alone	1500	0	0	1500	5.5
	+NaCN	1300	15	37	1352	6.0
	+NaCN/CO	590	3	37	630	16

[a] Normal assay conditions under 101 kPa of Ar and a 20-fold molar excess of wild-type Fe protein, with the addition of 5 mM NaCN or of 5 mM NaCN plus 10 kPa of CO. [b] Product formation expressed as specific activity in electron pairs·(min·mg of MoFe protein)$^{-1}$ appearing in each product. For CH_4 and NH_3 production, the listed specific activity must be divided by 1.5 for nmol of product·(min·mg of MoFe protein)$^{-1}$. The specific activities for NH_3 include a variable contribution from CH_3NH_2 formation (see text). All data are reported at most to two significant figures. [c] Represents the sum of the individual specific activity for each product. [d] Expressed as the number of MgATP molecules hydrolyzed for each electron pair found in measured products.

Nitrogenase Assays. Unless otherwise stated, assays were performed at a total protein concentration of 0.5 mg·mL^{-1} with a 20-fold molar ratio of wild-type Fe protein over either wild-type or altered MoFe protein at 30 °C in 9.25 mL reaction vials fitted with butyl rubber stoppers and crimped with aluminum caps. Each assay contained, in a reaction volume of 1 mL, 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 (25 units)

of creatine phosphokinase. Gaseous substrates and/or inhibitors were added by gastight syringe to a 100% argon atmosphere and the vials then vented to atmospheric pressure. MoFe protein was added, and after a 3 min incubation period at 30 °C, the reaction was initiated by addition of Fe protein. Alternatively, premixed nitrogenase components were used to initiate the reactions, which were terminated by injection of 0.25 mL 0.5 M EDTA-Na_2 (pH 7.5). All data points were collected in triplicate, and each experimental series was repeated at least twice.

Nitrogenase Assays Using NaCN. An anaerobic 100 mM NaCN stock solution was prepared. After flushing the desired amount of solid NaCN in a sealed serum vial with argon, degassed 25 mM HEPES (pH7.4) was added, followed by a predetermined amount of degassed 9 M HCl to obtain the desired pH. Appropriate aliquots of the stock solution were added by syringe to each anaerobic assay vial at the start of the temperature preincubation period, followed in sequence by the MoFe protein and the Fe protein or by a premixed combination of the two. Reactions were terminated by injection of 0.25 mL of 0.5 M EDTA-Na_2 (pH 7.5). In the experiments to investigate the effect of adding either C_2H_2 or C_2H_4 on both HCN reduction and CN^- inhibition, the appropriate amount of hydrocarbon was added to the vials directly after addition of the NaCN stock solution, followed by venting to atmospheric pressure. C_2H_2 was prepared by the action of H_2O on CaC_2. Contaminating C_2H_6 was removed from the C_2H_4 (Scott Specialty Gases, Inc., Plumsteadville, PA) before use as described by Ashby et al. (*36*).

The K_m for CH_4 production from HCN was determined for each of the four MoFe proteins by measuring the CH_4 evolved in assays containing 0.043 mg of MoFe protein at a 20:1 Fe protein:MoFe protein molar ratio over the range 0.02−60 mM NaCN at pH 7.4. Concentrations of HCN and CN^- were calculated using values of 7.24×10^{10} for the dissociation constant for HCN and 1.468×10^{-14} for water at 30 °C.

Analytical Methods. Dihydrogen evolution was measured by gas chromatography on a molecular sieve 5A column (Supelco, Bellefonte, PA) and a TC detector. Methane, ethylene, and ethane were quantified with a Porapak N column and FID detection. For assays at high C_2H_4 concentrations, a column of chromatographic alumina was used to separate C_2H_6 and C_2H_4 (*36*). The calibration gases used were 1000 ppm of CH_4 in He, 1000 ppm of C_2H_4 in He, 1000 ppm of C_2H_6 in He, and 1% H_2 in N_2 (Scott Specialty Gases, Inc., Plumsteadville, PA). Creatine, as a measure of MgATP hydrolysis, was determined by the method of Ennor (*37*) after the reaction vial contents had been passed through a 2.5 cm × 6 mm column of Dowex-1X2 (Cl^- form) (*38*). NH_3 *plus* methylamine was measured on an aliquot of this effluent by the phenol−hypochlorite method after adsorption and elution from a 1.5 cm × 6 mm column of Dowex 50W-X8 (Na^+ form) to remove creatine, which interferes with the color development (*39*).

To quantify ammonia and methylamine individually, a 38 mL serum vial, which contained the same concentrations of reactants in a 4 mL reaction volume, was used. After quenching with 1 mL of 0.5 M EDTA-Na_2 (pH 7.5), 4.5 mL of the vial contents was applied to a 4 cm × 6 mm Dowex-1X2 (Cl^- form) column and washed through with 1 mL of water. A 0.5 mL aliquot of the effluent was removed, and subsamples were used to quantify the creatine concentration. As a control, a 2 mL aliquot was applied to a 1.5 cm × 6 mm Dowex 50W-X8 column (Na^+ form), and total NH_3 *plus* CH_3NH_2 was determined as described before (*39*). The remaining 3 mL of the effluent was loaded onto a 4.5 cm × 6 mm column of Dowex 50W-X8 (Na^+ form), which was then washed with two 0.5-mL volumes of water and two 0.5-mL volumes of 0.25 M NaCl to remove creatine. The column was developed with up to 15 1-mL volumes of 10 mM sodium pyrophosphate buffer (pH 10.2 as a 0.1 M solution before dilution) containing 0.1 M NaCl. The actual number of washes was predetermined by using a control sample containing known amounts of NH_3 and CH_3NH_2. This procedure completely eluted the NH_3, which was quantified by the phenol−hypochlorite method (*38*). The CH_3NH_2 was then eluted into tubes, which contained 0.09 mL of 0.1 M HCl, with four-to-five 0.5-mL volumes of 19 mM NaOH containing 0.5 M NaCl, and assayed by the ninhydrin method (*40*), which was modified by making up the ninhydrin reagent in 0.4 M citrate buffer (pH 5.0) to accommodate a larger sample volume (0.35 mL) and a lesser dilution with 50% (v/v) propan-1-ol to increase sensitivity. A typical elution profile is illustrated in Figure 2. Recoveries of NH_3 and CH_3NH_2 from artificial mixtures in otherwise normal reaction mixtures containing 5 mM NaCN, but lacking enzyme, were 103.9 ± 6.1% and 97.6 ± 6.5%, respectively.

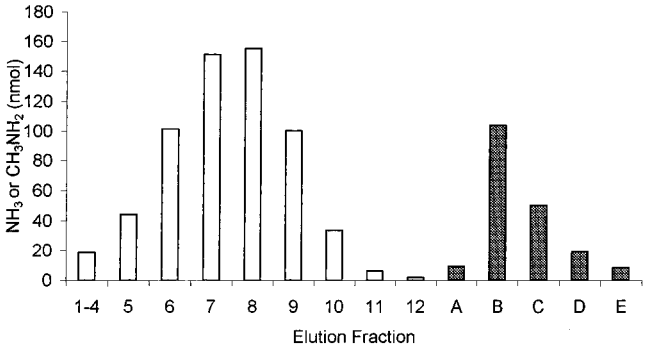

FIGURE 2: Elution profile of a pretreated reaction mixture from a 4.5 cm × 6 mm column of Dowex 50W-X8 (Na^+ form) developed with 12 1-mL volumes of 10 mM sodium pyrophosphate buffer to elute the NH_3 (open bars, 1−12), which was quantified by the phenol−hypochlorite method (*38*). CH_3NH_2 (hatched bars, A−E) was eluted with 5 0.5-mL volumes of 19 mM NaOH containing 0.5 M NaCl and assayed by a modified ninhydrin method (*40*).

Source of the Additional Methane. When C_2D_2 was used with wild-type nitrogenase, it was produced by adding D_2O (99.9%; Sigma, St. Louis, MO) to an appropriate amount of solid calcium carbide in an evacuated vessel as described previously (*33*). Assays were performed in argon-flushed flasks of about 125 mL capacity containing 2 kPa of C_2D_2 in the gas phase and 1 mM NaCN in solution. Separate titration experiments showed that CH_4 production by wild-type Mo-nitrogenase was maximal at 1 mM NaCN with 2 kPa of added C_2H_2 (data not shown). Each assay contained 900 μmol of creatine phosphate, 750 μmol of HEPES buffer (pH 7.4), 400 μmol of sodium dithionite, 150 μmol of $MgCl_2$, 75 μmol of ATP, and 3.75 mg (750 units) of creatine phosphokinase in a total liquid volume of 20 mL. Each assay was initiated by addition of MoFe protein (2 mg) and Fe protein (11.2 mg), incubated at 30 °C for 30 min in a shaking

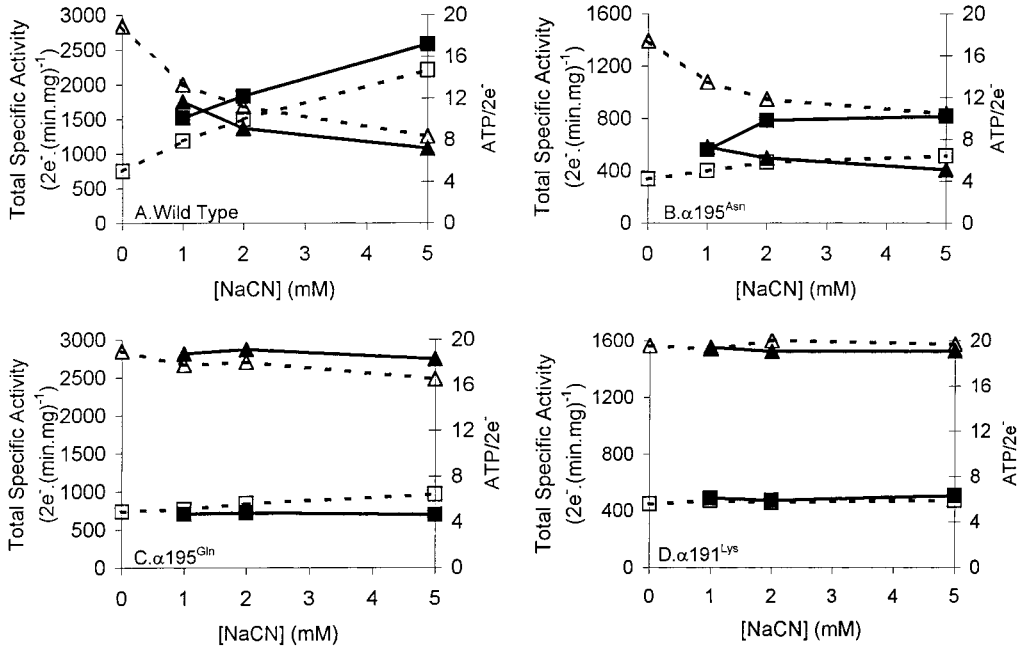

FIGURE 3: Total electron flux to all products (△, ▲) and the resulting ATP/2e⁻ ratio (□, ■) as a function of increasing NaCN concentration, in the absence (open symbols and dashed lines) and in the presence (filled symbols and solid lines) of 5 kPa of added C_2H_2, for (A) wild-type MoFe protein, (B) α-195^{Asn} MoFe protein, (C) α-195^{Gln} MoFe protein, and (D) α-191^{Lys} MoFe protein. The total specific activity was calculated as described in the Results section.

water bath, and terminated by addition of 5 mL of 0.5 M EDTA-Na_2 (pH 7.5). Gas samples (0.2 mL) were withdrawn for gas chromatographic analysis for C_2H_4, CH_4, and H_2. The liquid contents of the flask were then frozen by partial immersion in a dry ice−ethanol bath and the gaseous contents allowed to equilibrate through a gastight connector to a previously evacuated, 10 cm path-length, infrared gas cell of 100 mL capacity. The infrared spectrum of the gas was recorded on a MIDAC (Irvine, CA) Model M2004 Fourier transform infrared spectrometer with Grams 32 software.

RESULTS

Initial HCN Reduction Experiments. Table 1 shows that all four MoFe proteins were purified to relatively high specific activity and lists the production of CH_4 and NH_3 from catalyzed HCN reduction, plus concomitant H_2 evolution. CH_3NH_2, which is produced by wild-type *A. vinelandii* Mo-nitrogenase in a constant 0.35:1 molar ratio with CH_4 (*15*), was not measured in these initial experiments. With 5 mM NaCN, the rates of HCN reduction, as measured by CH_4 production, by the nitrogenases containing the α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins were 59%, 159%, and 6%, respectively, of the wild-type activity. Of the electron flux appearing as products, which is a combined measure of CH_4, NH_3, and H_2 production only in this instance, 51%, 50%, 33%, and 4%, respectively, were allocated to CH_4 *plus* NH_3 for the wild-type, α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins, respectively. Total electron flux in the presence of 5 mM NaCN was substantially decreased for both the wild-type and α-195^{Asn} MoFe proteins (Figure 3A,B). In contrast, total electron flux through both the α-195^{Gln} and the α-191^{Lys} MoFe proteins was only minimally affected (Figure 3C,D). Ignoring putative CH_3NH_2 production, total electron flux through the wild-type, α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins was inhibited by 64%, 49%, 12%, and 10%, respectively, by 5 mM NaCN. This decrease in total electron flux for the wild-type and α-195^{Asn} MoFe proteins was associated with a 2−3-fold increase in the ATP/2e⁻ ratio (Figure 3A,B) because the rate of MgATP hydrolysis was unaffected by adding 5 mM NaCN to assays involving any of the four MoFe proteins (data not shown).

Table 1 also shows that, when 10 kPa of CO was added to sodium cyanide-containing assays with nitrogenases incorporating the wild-type, α-195^{Asn}, or α-195^{Gln} MoFe proteins, electron flux was either fully recovered or slightly enhanced, HCN reduction was very significantly inhibited, and MgATP hydrolysis regained its tight coupling to electron flux. The exception, the nitrogenase incorporating the α-191^{Lys} MoFe protein, exhibited CO inhibition of total electron flux (*32*), which was reflected in an overall 61% decrease in the rate of H_2 evolution and the uncoupling of MgATP hydrolysis from electron flux to give an elevated ATP/2e⁻ ratio. In addition, although CH_4 production was virtually eliminated, the rate of NH_3 production was unaffected by CO.

In separate experiments (data not shown), the K_m value for CH_4 production from nitrogenase-catalyzed HCN reduction for each MoFe protein was calculated from the NaCN concentration at pH 7.4 as follows: wild type, 1.6 mM HCN; α-195^{Asn}, 0.45 mM HCN; α-195^{Gln}, 4.5 mM HCN; and α-191^{Lys}, 12 mM HCN.

Additional titration experiments showed that, in agreement with Li et al. (*15*), 5 mM NaCN maximized the rate of CH_4 production with wild type (data not shown). Accordingly, to facilitate comparisons, no higher NaCN concentrations were used with any of the MoFe proteins used in this study. A separate set of titrations investigated the effect of added C_2H_2 on the rates of production of both CH_4 and NH_3 from HCN in solutions of 1−5 mM NaCN. The C_2H_2 effect was maximal at 5 kPa for all of the nitrogenases (data not shown),

Table 2: Product Formation from HCN Reduction at 5 mM NaCN with and without 2 kPa of C_2H_2

MoFe protein[a]	C_2H_2 (kPa)	electron pairs appearing in products formed[b] H_2	CH_4	NH_3	CH_3NH_2	C_2H_4	C_2H_6	total SA[c]	ATP/$2e^-$ [d]
wild type	0	270	150	150	58			628	20
	2	100	140	130	76	130	0	576	22
α-195^{Asn}	0	220	120	150	71			561	8.1
	2	160	17	77	43	18	11	326	15
α-195^{Gln}	0	1400	400	420	150			2370	5.7
	2	1300	90	160	37	400	0	1987	6.5
α-191^{Lys}	0	1300	11	21	40			1372	6.0
	2	1200	13	20	17	17	5	1272	5.8

[a] Normal assay conditions, except that 4 mL total reaction volumes were used, under either 101 kPa of Ar or 2 kPa of C_2H_2/99 kPa of Ar, with a 20-fold molar excess of wild-type Fe protein and 5 mM NaCN at pH 7.4. [b] Product formation expressed as specific activity in electron pairs·(min·mg of MoFe protein)$^{-1}$ appearing in each product. For rates of product formation on a nanomole basis, these specific activities must be divided either by 1.5 for CH_4 and NH_3 production or by 2 for CH_3NH_2 formation. All data are reported at most to two significant figures. [c] Represents the sum of the individual specific activity for each product. [d] Expressed as the number of MgATP molecules hydrolyzed for each electron pair found in measured products.

and further additions of C_2H_2 above 5 kPa lowered only the rate of H_2 evolution. With wild-type nitrogenase, this lowered rate of H_2 evolution was not accompanied by an increased rate of C_2H_4 evolution and resulted in a decreased total electron flux. With the three altered nitrogenases, an increased rate of C_2H_4 (and C_2H_6, where appropriate) evolution did compensate for the lowered H_2 evolution rate at the higher C_2H_2 pressures.

Methylamine Production from HCN with or without C_2H_2. Methylamine production was measured for each of the four nitrogenases in the presence of 5 mM NaCN, plus or minus 2 kPa of C_2H_2. The results in Table 2 show that the presence of C_2H_2 had either little or no effect on the *relative* rates of CH_3NH_2 and CH_4 production by both the wild type and the nitrogenase incorporating the α-195^{Gln} MoFe protein. The ratio of these two rates (CH_3NH_2/CH_4) for wild type, on a molar basis, averaged out as 0.35:1, which is in excellent agreement with the previously reported value of 0.35:1, with and without C_2H_2 (*15*). For the α-195^{Gln} MoFe protein, the ratio averaged out as 0.30:1. In marked contrast, added C_2H_2 *increased* the ratio of these rates more than 4-fold for the nitrogenase containing the α-195^{Asn} MoFe protein but *decreased* this ratio of rates about 3-fold for the nitrogenase containing the α-191^{Lys} MoFe protein. The addition of C_2H_2, however, had a very different effect on the *absolute* rates for these nitrogenases. For example, at 5 mM NaCN, the rates of both CH_4 production and CH_3NH_2 production with wild-type nitrogenase are hardly affected by C_2H_2, whereas with the α-195^{Gln} nitrogenase, both rates are significantly and similarly inhibited. Both situations lead to no significant change in the ratio of the two rates. With the α-195^{Asn} nitrogenase, both rates are decreased by added C_2H_2, but the rate of CH_4 production is inhibited much more. With the α-191^{Lys} nitrogenase, only one of the two rates is changed by added C_2H_2; the rate of CH_4 production is hardly affected, whereas the rate of CH_3NH_2 production decreases significantly.

Product Formation as a Function of NaCN Concentration. The method (*39*) used to recover NH_3 for its assay with phenol−hypochlorite also recovers CH_3NH_2; the NH_3 data are consequently high. On a molar basis, CH_3NH_2 produces only 14% of the color yield of NH_3 (data not shown). This value, together with the determined CH_3NH_2:CH_4 molar ratio values (from data in Table 2) and the assumption that this ratio does not change with NaCN concentration, was used both to calculate specific activities for CH_3NH_2 production and to adjust the specific activities for NH_3 production from assays in which the NaCN concentration was varied between 0 and 5 mM. Each molecule of excess NH_3 was assigned two electrons (to account for the presumptive HCHO formation) compared with three electrons for each NH_3 molecule that was accompanied by formation of a CH_4 molecule (*15*). The adjustments to the NH_3 rate data are generally slight. Each CH_4, H_2, and CH_3NH_2 formed was assigned three, two, and four electrons, respectively. These values were then used to calculate the total specific activity for each nitrogenase from the electron pairs appearing in each product using the formula:

$$\sum(\text{electron pairs}) = [H_2] + [3 \times CH_4] + [NH_3 - CH_4] + [2 \times CH_3NH_2]$$

The trends of these total specific activity calculations, together with the resulting ATP/$2e^-$ ratios, are plotted in Figure 3 as a function of NaCN concentration in the absence and presence of 5 kPa of C_2H_2 and expand the data presented in Table 1.

The effects of NaCN concentration on the rates of formation of the individual products of HCN reduction are presented in Figure 4 under the same assay conditions. The rates of H_2 production, which decreased with increasing NaCN concentration, are not shown but can (in the absence of C_2H_2) be calculated from the difference between total specific activity (Figure 3) and the sum of the specific activities for formation of products shown in Figure 4. In the presence of C_2H_2, the calculation is complicated by the production of C_2H_4 (plus C_2H_6 for the α-195^{Asn} and α-191^{Lys} nitrogenases) from the added C_2H_2. However, for each nitrogenase, 5 kPa of added C_2H_2 resulted in a constant decrease in the rate of H_2 evolution, which was independent of the NaCN concentration. The rate of H_2 production decreased to 20%, 50%, 60%, and 95% of its C_2H_2-free value for the wild-type, α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} nitrogenases, respectively.

For the wild-type MoFe protein, the rates of formation of all HCN-derived products increased with increasing NaCN concentration. Either 5 mM NaCN or 5 kPa of C_2H_2 eliminated excess NH_3 production (Figure 4A) by equalizing the rates of both CH_4 and NH_3 production. Added C_2H_2 also exacerbated the inhibition of total electron flux, thereby increasing the ATP/$2e^-$ ratio (Figure 3A).

For the α-195^{Asn} MoFe protein, the rates of formation of NH_3 and CH_4 were maximal at 1 mM NaCN, but the rate of NH_3 production was much higher. Added C_2H_2 nearly eliminated CH_4 production, markedly inhibited the rate of NH_3 production, and further exacerbated the inhibition of total electron flux because the combined rates of C_2H_4 and C_2H_6 production did not compensate for the lost capacity for H_2 evolution and HCN reduction. Excess NH_3 was

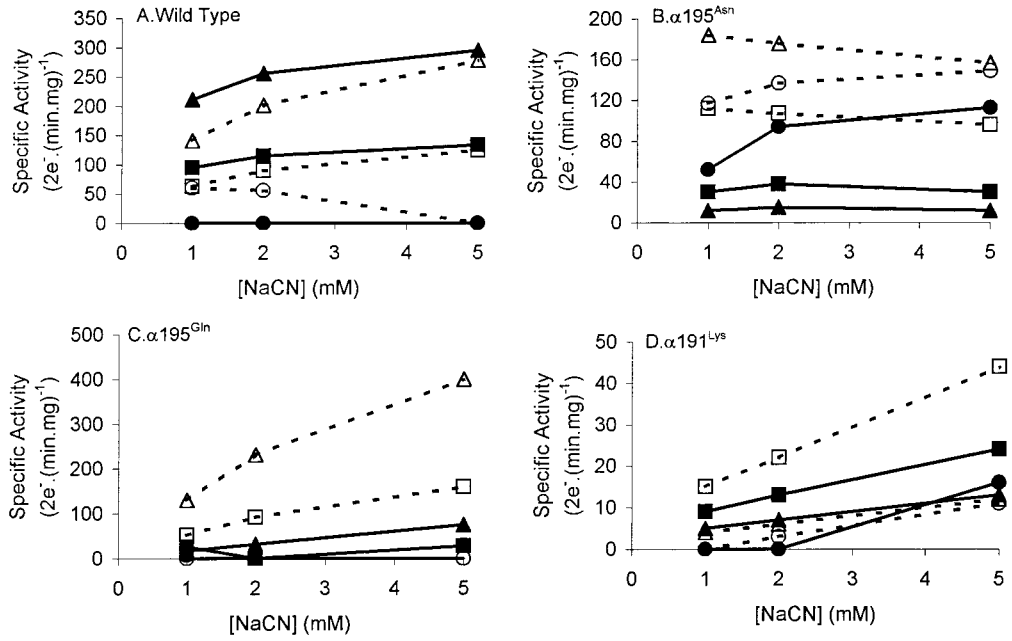

FIGURE 4: Specific activities of CH_4 formation (△, ▲), "excess NH_3" formation (○, ●), and calculated CH_3NH_2 formation (□, ■) as a function of increasing NaCN concentration, in the absence (open symbols and dashed lines) and in the presence (filled symbols and solid lines) of 5 kPa of added C_2H_2, for (A) wild-type MoFe protein, (B) α-195^{Asn} MoFe protein, (C) α-195^{Gln} MoFe protein, and (D) α-191^{Lys} MoFe protein. Because each molecule of CH_4 produced is accompanied by one molecule of NH_3, the total specific activity of NH_3 production is the sum of those for CH_4 and excess NH_3 production. The specific activity for CH_3NH_2 formation was calculated as a fixed proportion of CH_4 formation as described in the Results section.

Table 3: Summary of the Behavior of Wild-Type and Altered MoFe Proteins in Relation to HCN Reduction

property	C_2H_2 (5 kPa) present?	type of MoFe protein: wild type	α-195^{Asn}	α-195^{Gln}	α-191^{Lys}
rate of HCN reduction	no	high (100%)	moderate (60%)	highest (160%)	very low (6%)
at 5 mM NaCN	yes	high; unchanged	lower; especially CH_4	much lower	very low; unchanged
apparent affinity for HCN	no	K_m (CH_4) =	increased; K_m (CH_4) =	decreased; K_m (CH_4) =	decreased; K_m (CH_4) =
compared to wild type		1.6 mM HCN	0.45 mM HCN	4.5 mM HCN	12 mM HCN
increase in rates of CH_4 and	no	yes	no	yes	yes
NH_3 formation as NaCN	yes	yes	CH_4, no; NH_3, yes	yes	yes
increased from 1 to 5 mM?					
rate of NH_3 formation	no	higher; falling with	higher; unaffected by	equal	higher; unaffected by
compared to rate of CH_4		increase in HCN concn	increase in HCN concn		increase in HCN concn
formation	yes	equal	much higher	equal	higher
ratio of rates of CH_3NH_2	no	0.29	0.46	0.28	2.6
and CH_4 production	yes	0.40	1.9	0.30	1.4
inhibition of total electron	no	yes	yes; less than wild type	marginal	no
flux by CN^- ion?	yes	yes; exacerbated	yes; exacerbated	no	no

decreased, but not eliminated, by 5 kPa of added C_2H_2 (Figure 4B).

Although the rates of formation of both NH_3 and CH_4 by the α-195^{Gln} MoFe protein increased as the NaCN concentration increased (as observed for wild type), the rates were always equal with no excess NH_3 being produced (Figure 4C). Added C_2H_2 (5 kPa) may have induced minor excess NH_3 production. With this altered MoFe protein, the rate of C_2H_4 production compensated for the decreased reduction rates of both HCN and H^+.

The α-191^{Lys} MoFe protein reduced HCN poorly. It usually produced more NH_3 than CH_4 but, unlike wild type, failed to equalize the ratio between them at 5 mM NaCN. The addition of 5 kPa of C_2H_2 had almost no effect on HCN reduction and did not produce a wild-type-like increase in CH_4 production (Figure 4D). However, the resulting combined production of C_2H_4 and C_2H_6 did balance the decrease in H_2 evolution.

The responses of each of the four nitrogenases to NaCN concentration and to the addition of C_2H_2 are summarized and compared in Table 3.

Origin of the Increased CH_4 Production. Although reduction of C_2H_2 has never been reported to result in CH_4 production, we sought to ensure that HCN was indeed the source of enhanced CH_4 production in the presence of C_2H_2 by assaying wild-type nitrogenase in the presence of 2 kPa of C_2D_2 and 1 mM NaCN. After gas chromatographic analysis showed the expected enhancement of CH_4 production, the Fourier transform infrared spectrum of the gas showed bands at 1306 and 3106 cm^{-1}, both of which are attributable to CH_4. The infrared spectrum of a control gas mixture of 2 kPa of C_2D_2 with CDH_3 (Cambridge Isotope Laboratories, Andover, MA) at the same concentration as the CH_4 found in the incubation clearly showed a 2199 cm^{-1} band corresponding to the C−D stretching frequency of CDH_3. This band at 2199 cm^{-1} was not observed after

nitrogenase incubation with 1 mM NaCN and 2 kPa of C_2D_2, indicating that acetylene itself is not the source of the extra methane formed.

Effect of C_2H_4 on HCN Reduction. Similar experiments, but with 1 mM NaCN only, were performed with nitrogenases containing each of the four MoFe proteins under a 50 kPa C_2H_4/51 kPa Ar atmosphere (data not shown). This concentration of NaCN gives sufficient products for accurate quantification with an acceptable level of inhibition of total electron flux (see Figures 3 and 4). The choice of 50 kPa of C_2H_4 was dictated by the known high K_m values for C_2H_4 reduction for all four MoFe proteins (*33*). No change in the rate of either CH_4 or NH_3 formation was observed with the nitrogenases incorporating the wild-type, the α-191^{Lys}, or the α-195^{Gln} MoFe protein. The primary impact of adding C_2H_4 to assays with any of these MoFe proteins was through its known inhibition of total electron flux. Thus, compared to 1 mM NaCN alone, both the H_2 evolution and MgATP hydrolysis rates were decreased by 11%, which is an amount that is consistent with prior observations (*33*, *36*, *41*). In addition to the 11% loss of total electron flux, the nitrogenase incorporating the α-195^{Asn} MoFe protein also showed a 37% decrease in the rates of both CH_4 and NH_3 formation due to competition for electron flux by the added C_2H_4, which is reduced to C_2H_6. This observation is consistent with the lower K_m for C_2H_4 exhibited by the α-195^{Asn} MoFe protein compared to those of the other MoFe proteins (*33*).

DISCUSSION

Effect of Added C_2H_2 on HCN and CN^- Interactions with Wild-Type Nitrogenase. To gain insight into how added C_2H_2 enhanced HCN reduction to CH_4, we first investigated the response of wild-type nitrogenase to increasing concentrations of NaCN in the absence of C_2H_2. Our results are consistent with those of Li et al. (*15*) and show the tendency of the NH_3 production rate to be higher than the CH_4 production rate due to excess NH_3 production (putatively from CH_2═NH release and hydrolysis; see Scheme 1). In addition, the wild-type MoFe protein suffered the greatest inhibition of total electron flux with increasing NaCN concentration of all four MoFe proteins studied, suggesting that it has the highest affinity for CN^-. If excess NH_3 arises from the escape and subsequent hydrolysis of the CH_2═NH intermediate, the electron flux through nitrogenase should affect the extent to which this reaction occurs. At low electron flux, any intermediate in the six-electron reduction pathway from HCN should, by being longer lived, be increasingly likely to escape and be hydrolyzed. Indeed, wild-type MoFe protein produces more excess NH_3 at the lower flux resulting from a 1:1 molar ratio of Fe protein:MoFe protein ratio than with an 8:1 ratio (*15*). One might, therefore, expect excess NH_3 to increase whenever increasing NaCN concentrations result in decreasing electron flux. In fact, the exact opposite occurs with wild-type MoFe protein. Excess NH_3 becomes zero, i.e., the rates of CH_4 and NH_3 production become equal, at 5 mM NaCN where electron flux to HCN reduction is maximized. This outcome suggests that the higher NaCN concentrations somehow discourage the escape of CH_2═NH. Thus, the drastically decreased electron flux at higher NaCN concentrations may well lower the apparent affinity of the wild-type MoFe protein for HCN, resulting in poorer HCN binding and, consequently, less displacement of any intermediate from the site.

When C_2H_2 was added to assays containing wild-type nitrogenase, the rate of CH_4 production from HCN reduction was enhanced to equal the rate of NH_3 production, which was unchanged. Thus, the simultaneous binding of C_2H_2 and HCN enhances CH_4 production by eliminating excess NH_3 formation by somehow preventing CH_2═NH from escaping the site. Added C_2H_2 also exacerbated (by 14%) the inhibition of total electron flux, even at 5 mM NaCN, where there was no enhancement of the rate of CH_4 formation. This latter result differs from that of Li et al. (*15*), where C_2H_2 was reported not to inhibit total electron flux, although their average value for electron flux with added C_2H_2 was 8% lower than the average value without C_2H_2. It may well be that either the additional electron flux inhibition caused by the added C_2H_2 or its competition for electron flux for reduction (or both) decreases the overall electron flux to HCN reduction in much the same way as when the NaCN concentration is increased. The consequence would be a lowering of the affinity of the wild-type enzyme for HCN, retention of the CH_2═NH intermediate at the site, a decrease in excess NH_3 formation, and increased CH_4 production.

Our previous work (*33*) had suggested that C_2H_2 and C_2H_4 share a common binding site on nitrogenase. However, C_2H_4, even at a 10-fold higher concentration than used with C_2H_2, did not enhance CH_4 production from HCN. It did, however, decrease the rates of both H_2 evolution and MgATP hydrolysis by an additional 11% over that caused by NaCN alone. This result indicates that the electron flux inhibition effects of CN^- and C_2H_4 are not only separate but additive, and therefore, these inhibitors have separate binding sites. This conclusion is supported by the fact that inhibition of total electron flux by C_2H_4 is inherently different to that caused by CN^- (*33*).

Under all conditions, C_2H_2 reduction to C_2H_4 occurred at the expense of H_2 evolution and hardly diminished HCN reduction. This observation confirms that, for wild-type MoFe protein, HCN is reduced at a site that is apparently in a more oxidized state than the sites that evolve C_2H_4 and H_2 (*15*). The Lowe−Thorneley scheme (*41*) indicates that C_2H_2 binds to the E_2 state of the MoFe protein of nitrogenase, which is produced after two electrons and two protons have been accepted from the Fe protein, and that H_2 is also evolved from this state. HCN, therefore, must bind to either the E_0 (resting) or the E_1 (one-electron, one-proton) state. We prefer binding to the E_1 state because considerable evidence exists to show that no substrate binds to the E_0 state of the MoFe protein (summarized in ref *42*).

The single HCN/CN^- binding site hypothesis (*17*) suggests that CN^- acts as a total electron flux inhibitor until it is protonated to give the substrate, HCN. Thus, it predicts that any addition, which enhances the rate of product formation, should alleviate inhibition of total electron flux due to increasing the ratio of protonated substrate to deprotonated substrate. However, with wild-type MoFe protein, the binding of C_2H_2 must have little or no effect on the degree of protonation of bound substrate because the increase in the CH_4 production rate was substantially offset by the simultaneous loss of excess NH_3 production. The major impact of added C_2H_2 appears to result only from its inhibitory effects on electron flux.

Effects of the Amino Acid Substitutions on the Interactions with HCN and CN⁻. In the absence of C_2H_2, the α-195^{Asn} MoFe protein behaved similarly to wild type except for when its higher than wild-type apparent affinity for HCN and its lower than wild-type apparent affinity for CN^- come into play. For example, the rates of both CH_4 and NH_3 production remained unequal even at 5 mM NaCN, implying that excess NH_3 production from CH_2═NH release and hydrolysis occurs more readily than with wild-type MoFe protein. In addition, its lower apparent affinity for CN^- would cause a smaller decrease in total electron flux (see above) and consequently a smaller decrease in HCN binding affinity than occurs with wild type. This property, together with its higher apparent HCN affinity compared to that of wild type, would allow HCN to be a more effective competitor for the site on the α-195^{Asn} MoFe protein and would always displace some of the intermediate to produce excess NH_3.

When C_2H_2 was present, the α-195^{Asn} MoFe protein responded very differently to wild type. The rate of CH_4 production decreased and did so much more substantially than the rate of NH_3 production. In addition, a greater exacerbation of the inhibition of electron flux occurred than with wild type. Therefore, in contrast to wild type in which C_2H_2 binding effectively prevents CH_2═NH escape and subsequent hydrolysis, C_2H_2 binding to the α-195^{Asn} MoFe protein continues to allow intermediate escape and hydrolysis to excess NH_3. This observation is consistent with this altered enzyme's higher affinity for HCN and its lower affinity for C_2H_2 (*33*) compared to wild-type MoFe protein. The higher HCN affinity would allow continued HCN binding and displacement of the CH_2═NH intermediate because C_2H_2 would be less effective than with wild type at diverting electron flux from HCN reduction. The combined rates of C_2H_4 and C_2H_6 production from the added C_2H_2 effectively offset the decreased H_2 evolution rate. However, they do not compensate for the substantial decrease in the rates of product formation from HCN, which results in a further decline in total electron flux to products. These observations are compatible with an alteration of the HCN/CN^- equilibrium by bound C_2H_2 to increase the population of bound CN^- and decrease that of bound HCN.

In contrast to both the wild-type and α-195^{Asn} MoFe proteins, the nitrogenase containing the α-195^{Gln} MoFe protein catalyzes HCN reduction without producing excess NH_3 (the rates of CH_4 and NH_3 production are always equal) and without suffering significant CN^--induced inhibition of total electron flux. Why is there no substantial release of hydrolyzable intermediate from this altered MoFe protein? The answer again likely hinges on the apparent affinity of the α-195^{Gln} MoFe protein for HCN, which, in this case, is substantially lower than that for wild type. HCN would, therefore, be less likely to displace intermediates from the site on the α-195^{Gln} MoFe protein than from the site on either the wild-type or α-195^{Asn} MoFe protein. Excess NH_3 is less likely to be produced in such a situation, and none was observed.

When 5 kPa of C_2H_2 was present, the response of the nitrogenase incorporating the α-195^{Gln} MoFe protein was again completely unlike that of wild type and different to that of the α-195^{Asn} MoFe protein. Rather than enhancing the rate of CH_4 production, added C_2H_2 effectively eliminated all product formation from HCN. However, the marginal decrease in electron flux caused by NaCN was relieved. These results cannot be explained by C_2H_2 altering the bound HCN/CN^- equilibrium. Had this equilibrium changed such that a minimal amount of bound ligand was in the HCN form (to explain the very low substrate reduction rate), then most ligand should be bound as CN^- and result in substantial electron flux inhibition, which did not occur. A better explanation is that this loss of HCN reduction activity simply reflects the different affinities of this α-195^{Gln} MoFe protein for the two substrates. The α-195^{Gln} MoFe protein has a comparable affinity to wild type for C_2H_2 (*26*, *33*) but a lower affinity for HCN (this work). Thus, C_2H_2 may just outcompete HCN for the available electron flux under these conditions. If so, then, unlike wild type, HCN/CN^- and C_2H_2 may bind to the same oxidation state of the α-195^{Gln} MoFe protein.

Unlike the other MoFe proteins, the nitrogenase incorporating the α-191^{Lys} MoFe protein was a very ineffective catalyst for HCN reduction, consistent with its high (more than seven times that of wild type) K_m for CH_4 production. Surprisingly then, it produced excess NH_3. Possibly, the site's affinity for all intermediates is so low and all are held so loosely that escape is inevitable. Added C_2H_2 had no obvious effect on the rate of either CH_4 or NH_3 production by the α-191^{Lys} MoFe protein. This result is likely due its very high (70 times that of wild type; *33*) K_m for C_2H_2 reduction. C_2H_2 also did not change electron flux significantly. Therefore, it did not affect the HCN/CN^- equilibrium on the α-191^{Lys} MoFe protein.

Just as was found with wild type, C_2H_4 did not enhance CH_4 production from HCN with any of the three altered MoFe proteins. This result could simply reflect their 50−250-fold higher K_m values for C_2H_4 reduction compared to those for C_2H_2 reduction (*33*). However, C_2H_4 binding did occur and was manifested as an 11% inhibition of the rates of both H_2 evolution and MgATP hydrolysis, which is identical to that experienced by wild type with C_2H_4.

These studies show that the amino acid residues residing at positions α-191 and α-195 clearly influence the course of HCN reduction. They modulate the affinity of nitrogenase for HCN, the release of intermediates formed during its reduction, and the electron flux flowing to substrate and resulting in product. These residues also moderate the impact of simultaneously bound C_2H_2 on these same processes. Possible bases for these effects are discussed below under Structure−Function Insights.

Methylamine Production. The source of the variations in the rate of CH_3NH_2 formation by the four nitrogenases is more difficult to characterize. It could be that release of a fixed proportion of bound substrate as this four-electron-reduced product is a direct consequence of the enzymatic mechanism. The amount released could be a fixed percentage of the CH_3NH_2 level intermediate formed, the remainder of which would be committed to be reduced to CH_4 and NH_3. If so, then any added factor should affect the rates of formation of both products equally. The observed correlation among the rates of formation of the four-electron (CH_3NH_2) product and the six-electron (CH_4) product for wild type in the presence of either C_2H_2 or N_2O (*15*), neither of which significantly affected overall electron flux to all products, supports this possible explanation. Under conditions of

maximum electron flux to HCN reduction, i.e., at 5 mM NaCN, we found that added C_2H_2 has exactly this effect with both the wild-type and α-195^{Gln} MoFe proteins. With wild type, added C_2H_2 barely affects either total electron flux to all products or the specific electron flux that is used to reduce HCN. Thus, each product (CH_4, NH_3, and CH_3NH_2) of HCN reduction retains the same share of electron flux as under C_2H_2-free conditions. With the α-195^{Gln} MoFe protein, added C_2H_2 hardly changed total electron flux to products. However, it very substantially and about equally decreased electron flux to each of the products of HCN reduction. With the α-191^{Lys} MoFe protein, total electron flux to all products is again barely affected by added C_2H_2. However, there is a decrease in electron flux to CH_3NH_2 production, which is offset by formation of C_2H_4 and C_2H_6 from reduction of the added C_2H_2, with no concomitant decrease in electron flux to CH_4 production. Because only about 5% of the electron flux was used for HCN reduction, it is difficult to draw conclusions from this data set. Only with the α-195^{Asn} MoFe protein is total electron flux significantly inhibited by added C_2H_2 and only here does the presence of C_2H_2 impact the production of CH_4 and CH_3NH_2 differently. The proportion of electron flux going to CH_3NH_2 remains unchanged when C_2H_2 is added, whereas that going to the production of CH_4 is drastically decreased. Thus, these data suggest that CH_3NH_2 production is not necessarily correlated with CH_4 production but could be dependent on overall electron flux passing through each MoFe protein and, therefore, on the status of the equilibrium among the protonated (HCN) and deprotonated (CN^-) form of bound substrate.

Why and how CH_3NH_2 release occurs remains unclear, especially because the six-electron reduction of N_2 by Mo-nitrogenase does not release the four-electron product, N_2H_4. In this regard, Li et al. (*15*) suggest that the less favorable ΔH of −14 kcal/mol for the two-electron reduction of CH_3NH_2 to CH_4 plus NH_3 relative to the ΔH of −42 kcal/mol for reduction of N_2H_4 to two molecules of NH_3 could explain this difference. The ΔG values for the two reactions (ΔG = −20 kcal/mol vs −44 kcal/mol, respectively) also show the same trend and support this suggestion.

Effect of CO on both HCN Reduction and CN$^-$ Inhibition of Electron Flux. As expected, CO exerted its potent inhibitory effect on the wild-type, α-195^{Gln}, and α-195^{Asn} MoFe proteins by significantly decreasing product formation from HCN and diverting essentially all electron flux to H_2 production. CO also relieved electron flux inhibition by CN^-. However, with the α-191^{Lys} MoFe protein, adding CO did not block NH_3 production from HCN, even though it did decrease CH_4 production significantly. This result implies that, with the α-191^{Lys} MoFe protein, excess NH_3 formation and consequently the two-electron reduction of HCN to CH_2═NH is *insensitive* to CO. This result is the second example of a change in the effect of CO binding on substrate reduction by the α-191^{Lys} MoFe protein because H_2 evolution from this same altered MoFe protein is *sensitive* to CO. These responses are the exact opposite of those that occur with wild-type nitrogenase. Together with the generally low activity of α-191-substituted MoFe proteins, they attest to the importance of the α-191^{Gln} residue in nitrogenase catalysis. Moreover, they suggest that the binding of both CO and some (at least) reducible substrates occurs on (or near) the [$MoFe_3S_3$-homocitrate] subcluster of the FeMo-cofactor.

Structure−Function Insights. Molecular modeling has indicated that, although a glutamine at the α-195 position can still form a hydrogen bond to a central sulfide of the FeMo-cofactor as occurs with the native histidine, an asparagine cannot (*26*). Clearly, the altered character of the hydrogen bond formed by α-195^{Gln} is inadequate for normal reduction of N_2 or azide (*31*), but it does not prevent HCN or H^+ reduction. Even substitution with asparagine, where the hydrogen bond cannot form and N_2 is not reduced (*3*), prevents neither HCN reduction nor CN^--induced inhibition of electron flux. If α-195^{His} is necessary for protonation of N_2 and azide (*31*), it is clearly not necessary for proton delivery to HCN, which likely utilizes a different proton channel into the FeMo-cofactor. An interaction between this proton channel and the properties of the amino acid residue at the α-195 position, which could be disseminated through the cofactor framework, may determine the protonation−deprotonation status of HCN/CN^- at their binding site.

Where might this HCN-related proton channel come into contact with the FeMo-cofactor and where might HCN/CN^- bind? A clue comes from the observation that, unlike the other three MoFe proteins, the α-191^{Lys} MoFe protein was ineffective in HCN reduction as well as in N_2 reduction. It has been suggested that, with wild type, the homocitrate component of FeMo-cofactor may become monodentate at the Mo atom during enzyme turnover by dissociation of its *β*-carboxylate, thereby freeing up a site for substrate binding to Mo on the [$MoFe_3S_3$-homocitrate] subcluster (*43*). This suggestion has been expanded (*44*) to indicate both how homocitrate needs to rotate to produce this vacant site and how the freed *β*-carboxylate could be well placed to become protonated and, thus, a potential donor to bound substrate. Whether or not this proposal is correct, any substantial movement of homocitrate would be modulated by the residue at position α-191 because, in wild type, the amide N of the α-191^{Gln} residue is hydrogen-bonded to a terminal carboxylate O of homocitrate (*6*). This interaction would be strengthened when glutamine is substituted by lysine and any movement requiring scission of the stronger ionic lysinyl-homocitrate bond would be inhibited. In support of this suggestion, we find that all altered MoFe proteins with charged residues at position α-191 are incapable of N_2 reduction (unpublished data). This inability to either make or break a bond with homocitrate could be reflected in the low activity of the α-191^{Lys} MoFe protein toward HCN/CN^- and suggests a proton channel utilizing the α-191 residue−homocitrate combination.

The particular amino acid residue at position α-191 and the resulting interaction with homocitrate affect not only HCN/CN^- binding/reduction but also both C_2H_2 binding/reduction (*33*) and the catalytic impact of the inhibitor, CO, as well. The site that binds HCN/CN^-, however, must be separate from at least one of the two sites (*28*, *33*, *45*) which bind C_2H_2, because both substrates can be bound simultaneously. We speculate that HCN, C_2H_2, and CO, unlike N_2 and azide, either do not bind in the vicinity of the α-195^{His} residue (*31*) or do not depend on this residue for effective binding, protonation, and/or reduction. We further suggest that the residue occupying the α-191 position will have a critical impact on the binding, protonation, and/or reduction

of all nitrogenase substrates, whereas the residue at the α-195 position will play a less important, but still mechanistically insightful, role.

Finally, we note that the amide O of the α-191^{Gln} residue is hydrogen-bonded to the backbone NH of α-61^{Gly}, which is adjacent to the P cluster-ligating residue, α-62^{Cys}. These residues, therefore, form a potential electron-transfer pathway from the P cluster through homocitrate to the FeMo-cofactor and bound substrate. Such an arrangement could provide tight correlation between *both* the reduction *and* the protonation of bound substrate through the intermediacy of homocitrate.

ACKNOWLEDGMENT

All mutant strains used in this study were constructed in an earlier collaborative project with D. R. Dean (Virginia Tech).

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BI0001628