Azotobacter vinelandii Nitrogenases Containing Altered MoFe Proteins with Substitutions in the FeMo-Cofactor Environment: Effects on the Catalyzed Reduction of Acetylene and Ethylene†

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ABSTRACT: Altered MoFe proteins of *Azotobacter vinelandii* Mo-nitrogenase, with amino acid substitutions in the FeMo-cofactor environment, were used to probe interactions among C_2H_2, C_2H_4, CO, and H_2. The altered MoFe proteins used were the α-195^{Asn} or α-195^{Gln} MoFe proteins, which have either asparagine or glutamine substituting for α-histidine-195, and the α-191^{Lys} MoFe protein, which has lysine substituting for α-glutamine-191. On the basis of K_m determinations, C_2H_2 was a particularly poor substrate for the nitrogenase containing the α-191^{Lys} MoFe protein. Using C_2D_2, a correlation was shown between the stereospecificity of proton addition to give the products, *cis*- and *trans*-$C_2D_2H_2$, and the propensity of nitrogenase to produce ethane. The most extensive loss of stereospecificity occurred with nitrogenases containing either the α-195^{Asn} or the α-191^{Lys} MoFe proteins, which also exhibited the highest rate of ethane production from C_2H_2. These data are consistent with the presence of a common ethylenic intermediate on the enzyme, which is responsible for both ethane production and loss of proton-addition stereochemistry. C_2H_4 was not a substrate of the nitrogenase with the α-191^{Lys} MoFe protein and was a poor substrate of the nitrogenases incorporating either the wild-type or the α-195^{Gln} MoFe protein, both of which had a low V_{max} and high K_m (120 kPa). Ethylene was a somewhat better substrate for the nitrogenase with the α-195^{Asn} MoFe protein, which exhibited a K_m of 48 kPa and a specific activity for C_2H_6 formation from C_2H_4 10-fold higher than the others. Neither the wild-type nitrogenase nor the nitrogenase containing the α-195^{Asn} MoFe protein produced *cis*-$C_2D_2H_2$ when turned over under *trans*-$C_2D_2H_2$. These results suggest that the C_2H_4-reduction site is affected by substitution at residue α-195, although whether the effect is related to the substrate-reduction site directly or is mediated through disturbance of the delivery of electrons/protons is unclear. Ethylene inhibited total electron flux, without uncoupling MgATP hydrolysis from electron transfer, to a similar extent for all four *A. vinelandii* nitrogenases. This observation indicates that this C_2H_4 flux-inhibition site is remote from the C_2H_4-reduction site. Added CO eliminated C_2H_4 reduction but did not fully relieve its electron-flux inhibition with all four *A. vinelandii* nitrogenases, supporting the suggestion that electron-flux inhibition by C_2H_4 is not directly connected to C_2H_4 reduction. Thus, C_2H_4 has two binding sites, and the presence of CO affects only the site at which it binds as a substrate. When C_2H_2 was added, it also eliminated C_2H_6 production from C_2H_4 and also did not relieve electron-flux inhibition fully. Thus, C_2H_2 and C_2H_4 are likely reduced at the same site on the MoFe protein. Two schemes are presented to integrate the results of the interactions of C_2H_2 and C_2H_4 with the MoFe proteins.

Nitrogenase is the catalyst in biological systems that "fixes" the otherwise unusable atmospheric dinitrogen and produces assimilable ammonia. The most commonly encountered form of this enzyme is Mo-nitrogenase, which consists of two metalloproteins, called the Fe protein and the MoFe protein. During substrate reduction catalyzed by wild-type Mo-nitrogenase, the Fe protein binds two molecules of MgATP and performs the role of a specific electron donor to the MoFe protein, the larger component protein. The MoFe protein contains the small-molecule-binding sites, which are located on the FeMo-cofactor, one of two types of metal-containing prosthetic groups found within the MoFe protein. The other is the P cluster, which is likely involved in accepting electrons from the Fe protein and transmitting them to the FeMo-cofactor. The interactions of the component proteins involve an association−dissociation cycle accompanied by hydrolysis of a minimum of two molecules of MgATP for every electron transferred. An often-used measure of the efficiency of substrate reduction is the ATP/$2e^-$ ratio, which is an index of the number of molecules of

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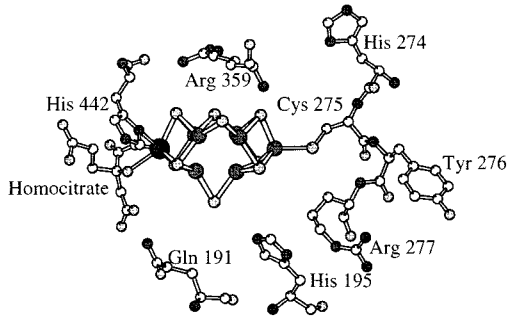


FIGURE 1: FeMo-cofactor and selected residues in its immediate vicinity (*12, 13*). Only two of the residues shown, α-275^{Cys} and α-442^{His}, are directly bonded to the FeMo-cofactor. Homocitrate (on the left) provides two bonds to the Mo atom, which is shown as the largest and darkest sphere, and also forms an −O ↓ HN− hydrogen bond with the amide function of α-191^{Gln} (at lower left). A −NH → S− hydrogen bond is formed between the ϵ-N of the imidazole ring of α-195^{His} and one of the three central sulfides that hold the two subclusters of the FeMo-cofactor together. The Fe atoms of the FeMo-cofactor are represented by spheres of intermediate size and shade, whereas the S atoms are smaller and lighter spheres. Elsewhere, the C atoms are unshaded, the O atoms are slightly darker, and the N atoms are of the darkest shade. The figure was drawn using MOLSCRIPT (*54*).

MgATP hydrolyzed for each pair of electrons appearing as product. An ATP/2e$^-$ ratio of 4−5/1 is usually measured with wild-type Mo-nitrogenase.

In the presence of a reductant of sufficiently low potential, an anaerobic environment, and a MgATP[1]-regenerating system, wild-type Mo-nitrogenase catalyzes the reduction of a number of alternative substrates in addition to N_2. Of these, proton reduction to H_2 (*1*) and C_2H_2 reduction to C_2H_4 (*2*) are the most commonly monitored. With wild-type *Azotobacter vinelandii* Mo-nitrogenase (*3*) but not with *Clostridium pasteurianum* Mo-nitrogenase (*4*), concentrations of C_2H_2 greater than 40 kPa inhibit electron flux and so lead to lower rates of C_2H_4 formation. Ethylene can also be reduced to ethane but with very low specific activity (*5*). The addition of two protons to the acetylene molecule occurs with a primarily cis stereochemistry (*2, 6*−*8*). CO is a noncompetitive inhibitor of the catalyzed reduction of all substrates except the proton (*9, 10*). Added CO diverts all electron flux to H_2 evolution so that neither the rate of electron flux nor the rate of MgATP hydrolysis is affected, resulting in an unchanged ATP/2e$^-$ ratio. In contrast, added H_2 inhibits only the N_2-fixation reaction.

Both component proteins of Mo-nitrogenase have been crystallized and their three-dimensional structures solved (*11*−*18*). That work has defined the structure, positioning, and bonding of the FeMo-cofactor within the MoFe protein (Figure 1). Further, because the evidence is compelling that the FeMo-cofactor is the site of substrate binding and reduction (*19*−*22*), this information allows the catalytic surface of the FeMo-cofactor to be logically probed. We have sought to gain insight into the spatial relationships among the binding sites of various nitrogenase substrates and inhibitors by disrupting precisely defined, localized areas of the FeMo-cofactor's environment through directed amino acid substitutions.

Amino acid sequence conservation in a number of Mo-nitrogenases was used to target certain residues in the MoFe protein (*23*). These residues were suggested to be either bound directly to the FeMo-cofactor or sufficiently close to influence its orientation or electronic properties (*21, 23*−*30*). Two obvious targets were the α-subunit residues, histidine-195 and glutamine-191. Substitution of either of these residues was predicted to modify both the substrate/inhibitor-binding and spectroscopic properties of the resulting altered nitrogenases. These suggestions found support in the crystal-structure data, but neither residue is covalently linked to the FeMo-cofactor. α-Histidine-195 approaches to within 3.2 Å (*31*) and forms a putative hydrogen bond to one member of the triangle of sulfides that form the "waist" of the FeMo-cofactor. α-Glutamine-191 engages in putative hydrogen-bonding with the shorter arm of the (*R*)-homocitrate (*32*) entity, which is otherwise ligated to the Mo atom of the FeMo-cofactor by its β-hydroxyl and β-carboxylate groups.

Substitution of the α-histidine-195 residue individually by a number of other amino acids (Asn, Tyr, Gln, Leu, Thr, or Gly) led to a variety of altered MoFe proteins (*21, 22*). None of these MoFe proteins, when complemented by the Fe protein, were able to reduce N_2 at appreciable rates, but they still catalyzed both proton reduction to H_2 and C_2H_2 reduction to C_2H_4 either alone or with some accompanying C_2H_6. Analysis of the catalytic and spectroscopic properties of these altered MoFe proteins has led to the suggestion that the α-histidine-195 residue helps to orient correctly the FeMo-cofactor within the MoFe protein so that N_2 binding can occur (*22, 33*). The MoFe protein in which the α-histidine-195 has been replaced with glutamine (abbreviated α-195^{Gln} MoFe protein) has N_2 behaving primarily as a reversible inhibitor of electron flux through the MoFe protein (*22*). However, the α-195^{Gln} MoFe protein does reduce N_2 to NH_3 but at only 1−2% of the wild-type rate (*34*). The resulting inhibition by N_2 of electron flux to substrate is not accompanied by an inhibition of the rate of MgATP hydrolysis. The α-195^{Gln} MoFe protein is also differentiated from the wild type in that it is essentially unaffected by the presence of CN^-, which in the wild type acts as a potent inhibitor of electron flux to substrate (*34*). In contrast, the α-195^{Gln} MoFe protein closely resembles wild type in its interactions with the substrates, H^+ and acetylene, and with the inhibitor, CO (*22, 34*).

Although the α-glutamine-191 residue is situated only one turn of a helix away, the phenotypes of mutant strains substituted at the α-glutamine-191 are very different from those of the α-histidine-195 mutants. Unlike the α-histidine-195 mutants, 5 of the 13 mutant strains constructed were able to grow diazotrophically. Also, when complemented with wild-type Fe protein, most α-glutamine-191-substituted MoFe proteins exhibit H_2 evolution, which is sensitive to varying extents to the presence of CO. For all the α-glutamine-191-substituted Mo-nitrogenases, C_2H_2 is a relatively poor substrate. Even so, both the α-191^{Lys} and α-191^{Glu} MoFe proteins produce small amounts of C_2H_6 as a product of C_2H_2 reduction (*21, 28*).

[1] Abbreviations: MoFe protein, the molybdenum- and iron-containing protein of nitrogenase; Fe protein, the iron-containing protein of nitrogenase; 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.

Table 1: Specific Activity and K_m for C_2H_2 Reduction and for C_2H_4 Reduction of MoFe Proteins from the Wild Type and Mutant Strains

	101 kPa of Ar[a]	10 kPa of C_2H_2/91 kPa of Ar[b]				
substitution	H_2	H_2	C_2H_4	C_2H_6	$K_m(C_2H_2)$[c]	$K_m(C_2H_4)$[d]
wild type	2820	240 (8%)	2610 (92%)	0	0.5	120
α-191^{Lys}	1630	1325 (91%)	105 (7%)	30 (2%)	35	nb[e]
		452[f] (42%)	488[f] (45%)	138[f] (13%)		
α-195^{Gln}	2860	1115 (45%)	1350 (55%)	0	0.5	120
α-195^{Asn}	1470	355 (31%)	535 (46%)	270 (23%)	1.0	48

[a] Specific activities are expressed as electron pairs (min·mg of MoFe protein)$^{-1}$ appearing as H_2 in the presence of a 20-fold molar excess of wild-type Fe protein. [b] Specific activities are expressed as electron pairs (min·mg of MoFe protein)$^{-1}$ appearing as each product. Therefore, for C_2H_6 production, the specific activity must be divided by 2 to obtain nmol of product (min·mg of MoFe protein)$^{-1}$. The numbers in parentheses are the percentage of electron-pair flux to each product. [c] K_m is expressed in kPa for the catalyzed production of C_2H_4 from C_2H_2. [d] K_m is expressed in kPa for the catalyzed production of C_2H_6 from C_2H_4. [e] nb indicates that C_2H_4 is neither bound nor reduced. [f] Specific activities in electron pairs (min·mg of MoFe protein)$^{-1}$ under 101 kPa of C_2H_2.

This obvious differentiation among the various substrates and inhibitors by these two sets of altered MoFe proteins suggests that the substitutions, by impacting different parts of the FeMo-cofactor's structure, are differentially altering its interaction with these substrates and inhibitors. A logical extension of this observation is that different substrates and inhibitors bind to different parts (sites) of the FeMo-cofactor. The α-195^{Asn}, α-195^{Gln}, and α-191^{Lys} MoFe proteins offer an opportunity to test some aspects of this hypothesis and the interactions among substrates and inhibitors. Here, we report our studies using H^+, C_2H_2, or C_2H_4 as substrate and CO or H_2 as inhibitor. We show that these altered MoFe proteins function very differently from one another and from the wild type, and the results provide important insights into the binding of substrates and inhibitors to the FeMo-cofactor.

EXPERIMENTAL PROCEDURES

Cell Growth and Protein Purification. Wild-type (α-191^{Gln}/α-195^{His}), DJ255 (α-191^{Lys}/α-195^{His}), DJ178 (α-191^{Gln}/α-195^{Asn}), and DJ540 (α-191^{Gln}/α-195^{Gln}) strains of *A. vinelandii* were grown in a 24 L fermentor at 30 °C in a modified, liquid Burk medium (*35*). Nitrogenase derepression and cell-extract preparation were performed as previously described (*28*). Cell extracts were heat treated for 5 min at 50 °C and cooled before being centrifuged at 98000*g* for 90 min. Nitrogenase component proteins were separated by Q-Sepharose anion-exchange chromatography using a linear NaCl-concentration gradient. The Fe protein was applied to a second Q-Sepharose anion-exchange column and purified to homogeneity. Purified Fe protein had a specific activity of 2800 nmol of H_2 produced (min·mg)$^{-1}$. The wild-type and altered MoFe proteins were further purified by gel filtration on Sephacryl S-200 before phenyl-Sepharose hydrophobic-interaction chromatography as previously described (*22*). The purified component proteins were concentrated using an Amicon microfiltration cell concentrator contained in an ice−water bath. Buffer exchange into 25 mM HEPES, pH 7.4, 10 mM $MgCl_2$ was carried out by passage through an anaerobic P-6DG column. Their specific activities are listed in Table 1. SDS−PAGE (12% polyacrylamide containing 1.35% cross-linker with a 4% stacking gel) with Coomassie Blue staining was used to confirm that all proteins were homogeneous (*36*). Unless otherwise stated, all buffers were saturated with argon and contained 2 mM sodium dithionite. Protein concentrations were determined by the method of Lowry et al. (*37*) and the Mo and Fe content of the proteins by inductively coupled plasma atomic emission spectroscopy using a Perkin-Elmer Plasma 400 spectrometer (Norwalk, CT).

Nitrogenase Assays. All activities of the wild-type and altered MoFe proteins were measured at 30 °C with a 20-fold molar excess of wild-type Fe protein, unless otherwise stated. Assays contained 0.5 mg of total nitrogenase proteins in 1 mL to avoid kinetic complications arising from either high- or low-protein concentrations. Assays were conducted at 30 °C in 9.25 mL reaction vials fitted with butyl rubber septa held by aluminum caps. Each assay contained, in a final volume of 1.0 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 of creatine phosphokinase. Gaseous substrates and/or inhibitors were added by gastight syringe to a 101 kPa argon atmosphere and then vented to atmospheric pressure. Unless otherwise stated, MoFe protein was added, and after a 3 min incubation period at 30 °C, the reaction was initiated by addition of Fe protein. Reactions were terminated, usually after 8 min, by injection of 0.25 mL of 0.5 M EDTA-Na_2, pH 7.5. The creatine released from creatine phosphate as the ATP is recycled during the assay was measured spectrophotometrically by the method of Ennor (*38*) with the modification described by Dilworth et al. (*39*).

Gaseous products were measured by gas chromatography. For evolved dihydrogen, a molecular sieves 5A column (Supelco, Bellefonte, PA) and a TCD detector were used, whereas acetylene, ethylene, and ethane were quantified with a Porapak N column and a FID. Under assay conditions employing high C_2H_4 concentrations, it was necessary to employ a column of chromatographic alumina to fully separate C_2H_6 and C_2H_4 (*5*). Calibration gases used were 1000 ppm C_2H_4 in He, 1000 ppm C_2H_6 in He, and 1% H_2 in N_2 (Scotty Specialty Gases, Plumsteadville, PA).

Assays under hyperbaric pressures of C_2H_4, which was purified as previously described (*4*), were performed in the same vials fitted with the same butyl rubber septa and aluminum seals. Just before the temperature-incubation stage, the argon atmosphere was replaced with C_2H_4 by flushing the vial with 7 volumes of C_2H_4. Immediately after injection of an appropriate mixture of Fe protein and MoFe protein, the vials were pressurized by injection of additional C_2H_4, e.g., an added 8.25 mL produced 202 kPa of pressure. Vials were then vented to atmospheric pressure after the desired incubation time and immediately quenched with 0.25 mL of 0.5 M EDTA-Na_2, pH 7.5. The contents of the headspace

were then analyzed by gas chromatography as described above.

Stereospecificity of C_2D_2 Reduction. C_2D_2 was generated by the addition of anaerobic D_2O to a predetermined amount of CaC_2 in a 25 mL crimp-sealed and evacuated serum vial. Typically, 70 mg of CaC_2 was weighed into a 25 mL vial, which was then capped and filled with argon at atmospheric pressure after four cycles of evacuation and filling. A 0.5 mL aliquot of D_2O (99.9% purity, Sigma) was then added and the reaction allowed to proceed to completion for 1 h. Prior experiments had shown that this combination would produce approximately 24 mL of C_2D_2. Initial infrared spectroscopic analysis (see below) indicated that the product contained less than 1% C_2DH. A 5 mL aliquot of gas was then removed and transferred by gastight syringe to an assay flask under argon. The assay flask was vented to atmospheric pressure after 5 min at 30 °C, and the resulting C_2D_2 concentration (4 kPa) was determined by gas chromatography. Each assay flask was of about 125 mL capacity with a sidearm that held a stopcock and a ground-glass adapter. Each assay contained 300 μmol of creatine phosphate, 50 μmol of $MgCl_2$, 25 μmol of ATP, 1.25 mg of creatine phosphokinase, 250 μmol of HEPES buffer, pH 7.4, and 200 μmol of sodium dithionite in a final total volume of 10 mL. A blank flask, with no added nitrogenase, was quenched with 2.5 mL of 0.5 M EDTA-Na_2, pH 7.5, immediately after venting. Each assay was initiated by addition of approximately 10 mg (in 0.2−0.5 mL) of a 20/1 molar mixture of wild-type Fe protein and either wild-type or altered MoFe protein. Assays were incubated at 30 °C for 10−30 min, depending upon the innate activity of each of the MoFe proteins, after which time they were terminated by addition of 2.5 mL of 0.5 M EDTA-Na_2, pH 7.5. Gas samples (0.1−0.2 mL) were then removed for gas-chromatographic analyses for C_2H_4, C_2H_6, and H_2. The liquid contents of the flask were frozen by partial immersion in a dry ice/ethanol bath and the gaseous contents then allowed to equilibrate through the gastight connector to a previously evacuated infrared gas cell of 100 mL capacity. The amounts of *cis*- and *trans*-$C_2D_2H_2$ and C_2DH_3 produced were estimated by Fourier transform infrared spectroscopy, using the height of the bands at 843, 988, and 943 cm^{-1}, respectively. Either a Perkin-Elmer (Norwalk, CT) model PE 1720-X spectrometer or a MIDAC (Irvine, CA) model M2004 spectrometer with Grams 32 software was used. Because the trans isomer has a molar absorptivity at 988 cm^{-1} of about half that of the cis isomer at 843 cm^{-1} (*8*), its measured height was doubled.

A similar protocol was used for the α-191^{Lys} MoFe protein, except that the assay flask was filled to 101 kPa with C_2D_2. This procedure involved evacuating the flask and connecting it directly to a similar-size, evacuated, C_2D_2-generation flask, which contained sufficient CaC_2 to generate about 101 kPa of C_2D_2 in the connected flasks. The appropriate amount of D_2O was then added cautiously to the carbide and the reaction allowed to proceed to completion. The pressure in the system was adjusted to 101 kPa, using a Hg manometer, by adding water to the C_2D_2-generation flask. The assay was started with a mixture of 3 mg of α-191^{Lys} MoFe protein and 16.4 mg of wild-type Fe protein and run for 20 min before quenching.

Reactions with trans-$C_2D_2H_2$. *trans*-$C_2D_2H_2$ (98% purity, Cambridge Isotope Laboratories, Andover, MA) was used as received. Initial infrared analysis showed no peaks at 843 and 943 cm^{-1}, indicating the complete absence of both the *cis* isomer and C_2DH_3, respectively. Reactions were performed in 70 mL two-necked flasks. One neck contained a ground-glass stopcock, and the other was closed with a butyl rubber septum. After receiving 15 mL of a mixture containing 450 μmol of creatine phosphate, 75 μmol of $MgCl_2$, 37.5 μmol of ATP, 1.88 mg of creatine phosphokinase, and 375 μmol of HEPES buffer, pH 7.4, each flask was evacuated and refilled with argon four times before 300 μmol of sodium dithionite was added. Each flask was then fully evacuated, and 55 mL (101 kPa) of *trans*-$C_2D_2H_2$ was injected by gastight syringe. The reactions were initiated by addition of 8 mg of wild-type Fe protein together with 1.5 mg of either wild-type or α-195^{Asn} MoFe protein. After either 30 min (α-195^{Asn} MoFe protein) or 15 min (wild-type MoFe protein) at 30 °C, reactions were terminated by addition of 4 mL of 0.5 M EDTA-Na_2, pH 7.5. Samples (0.1−0.2 mL) were withdrawn for gas-chromatographic analysis for C_2H_6 and H_2. The liquid contents of the flask were frozen and the gaseous contents analyzed by Fourier transform infrared spectroscopy as described above for the presence of *cis*- and *trans*-$C_2D_2H_2$ and C_2DH_3 using a MIDAC (Irvine, CA) model M2004 spectrometer with Grams 32 software.

RESULTS

MoFe Protein Purification and Substrate-Reduction Specific Activities. By careful selection of fractions eluting from both the Q-Sepharose and Sephacryl S-200 columns before application of phenyl-Sepharose hydrophobic-interaction chromatography, all four MoFe proteins used in this study were purified to high specific activity (Table 1). The metal content, reported as both mol of Mo/mol of MoFe protein and the iron-to-molybdenum ratio, of the four purified MoFe proteins was (wild type) 1.90 Mo, Fe/Mo = 13/1, (α-191^{Lys}) 0.90 Mo, Fe/Mo = 14/1, (α-195^{Gln}) 1.90 Mo, Fe/Mo = 13/1, (α-195^{Asn}) 0.85 Mo, Fe/Mo = 14/1. These values indicate that only approximately 50% of both the α-191^{Lys} and the α-195^{Asn} MoFe protein preparations are holo-MoFe protein. Although the nitrogenase with the α-195^{Gln} MoFe protein is a very poor N_2 fixer (*34*) and the nitrogenases with either the α-195^{Asn} or the α-191^{Lys} MoFe protein cannot reduce N_2 at all (*21*), all three purified MoFe proteins had very significant H_2-evolution activity under normal assay conditions. However, nitrogenases containing any of the three altered MoFe proteins catalyzed the reduction of C_2H_2 less effectively when compared to the wild type, which allocates 90% of its total electron flux for substrate reduction to C_2H_4 production under a 10 kPa of C_2H_2 in argon atmosphere. In contrast, when the α-195^{Gln}, α-195^{Asn}, or α-191^{Lys} MoFe proteins were used, only 55%, 69%, or 9%, respectively, of total electron flux was allocated to the combined production of C_2H_4 and C_2H_6 under the same conditions. Only those nitrogenases, which incorporated either the α-195^{Asn} or α-191^{Lys} MoFe protein, catalyzed the production of C_2H_6 from C_2H_2. Ethane production accounted for 23% and 2% of the total electron flux, but 34% and 21% of the electron flux allocated to C_2H_2 reduction, respectively.

Listed in Table 1 is the K_m for C_2H_4 production from C_2H_2 for each of the four MoFe proteins, when complemented with wild-type Fe protein. The K_m for α-195^{Gln} MoFe protein is identical to that of wild-type MoFe protein, but it diverts

Table 2: Production of C_2H_6 from C_2H_2 and of *trans*-$C_2D_2H_2$ from C_2D_2[a]

substitution	ethane[b] (as % of total product)	*trans*-$C_2D_2H_2$[c] (as % of total ethylene)	substitution	ethane[b] (as % of total product)	*trans*-$C_2D_2H_2$[c] (as % of total ethylene)
wild type	0	4	α-195^{Gln}	0	1
α-191^{Lys}	13	21	α-195^{Asn}	23	37

[a] All assays were performed at a 20/1 Fe protein/MoFe protein ratio in 10 mL total liquid volume contained in a 125 mL capacity flask. [b] The atmosphere was 10 kPa of C_2H_2/91 kPa of Ar except for α-191^{Lys}, where 101 kPa of C_2H_2 was used. [c] The atmosphere was 4 kPa of C_2D_2/97 kPa of Ar except for α-191^{Lys}, where 101 kPa of C_2D_2 was used.

Table 3: Catalyzed C_2H_4 Reduction: Effects of CO, C_2H_2, and H_2 on H_2 Evolution, C_2H_6 Formation, ATP/2e$^-$ Ratio, and Total Electron Flux

assay conditions	SPA[a] H_2	SPA[a] C_2H_6	flux[b]	ATP/2e$^-$
Wild-type MoFe Protein				
101 kPa of Ar	2730	0	2730	4.4
50 kPa of C_2H_4 + 51 kPa of Ar	2310	11	2321	4.4
50 kPa of C_2H_4 + 10 kPa of CO + 41 kPa of Ar	2595	0	2595	4.2
50 kPa of C_2H_4 + 10 kPa of C_2H_2 + 41 kPa of Ar	325	1	nd[c]	nd
50 kPa of C_2H_4 + 51 kPa of H_2	nd	18	nd	nd
α191^{Lys} MoFe Protein				
101 kPa of Ar	1530	0	1530	4.9
50 kPa of C_2H_4 + 51 kPa of Ar	1395	0	1395	5.5
50 kPa of C_2H_4 + 10 kPa of CO + 41 kPa of Ar	590	0	590	14.6
50 kPa of C_2H_4 + 10 kPa of C_2H_2 + 41 kPa of Ar	1205	25	nd	nd
50 kPa of C_2H_4 + 51 kPa of H_2	nd	2	nd	nd
α195^{Gln} MoFe Protein				
101 kPa of Ar	2765	0	2765	4.3
50 kPa of C_2H_4 + 51 kPa of Ar	2230	21	2251	4.5
50 kPa of C_2H_4 + 10 kPa of CO + 41 kPa of Ar	2405	1	2406	4.1
50 kPa of C_2H_4 + 10 kPa of C_2H_2 + 41 kPa of Ar	975	3	nd	nd
50 kPa of C_2H_4 + 51 kPa of H_2	nd	24	nd	nd
α195^{Asn} MoFe Protein				
101 kPa of Ar	1465	0	1465	4.5
50 kPa of C_2H_4 + 51 kPa of Ar	1140	75	1215	4.6
50 kPa of C_2H_4 + 10 kPa of CO + 41 kPa of Ar	1295	0	1295	4.7
50 kPa of C_2H_4 + 10 kPa of C_2H_2 + 41 kPa of Ar	330	157	nd	nd
50 kPa of C_2H_4 + 51 kPa of H_2	nd	83	nd	nd

[a] SPA is specific activity and is expressed as electron pairs (min·mg MoFe protein)$^{-1}$ appearing as product and not as nmol of product (min·mg of MoFe protein)$^{-1}$. [b] Flux is the overall rate of product formation, i.e., the total number of electron pairs (min·mg of MoFe protein)$^{-1}$ appearing in all products. [c] nd represents not determinable because the presence of one substrate masks the product of another substrate.

45% of its electron flux to H_2 evolution under 10 kPa of C_2H_2 compared to less than 10% for the wild type. For both the α-195^{Asn} and α-191^{Lys} MoFe proteins, the ratio of C_2H_4 to C_2H_6 produced was invariant throughout the K_m determinations; therefore, the K_m for C_2H_6 production from C_2H_2 must be the same as for C_2H_4 production from C_2H_2. The K_m for both C_2H_4 and C_2H_6 production from C_2H_2 for the α-191^{Lys} MoFe protein was 35 kPa. When the C_2H_2 concentration was increased to 101 kPa (3 times the K_m), the α-191^{Lys} MoFe protein produced C_2H_6 at 6 times the rate under 10 kPa of C_2H_2 and at about half the rate of the α-195^{Asn} MoFe protein under 10 kPa of C_2H_2. Under these conditions, 58% of the total electron flux was used for hydrocarbon products. Ethane accounted for 13% of the total electron flux but still represented 22% of the electron flux specific for C_2H_2 reduction.

Stereospecificity of C_2D_2 Reduction. Exposure of three of the four MoFe proteins individually to 4 kPa of C_2D_2 in argon (4−8 times their K_m) under turnover conditions was used to detect the stereospecificity of proton addition across the acetylenic triple bond. C_2D_2 (101 kPa; 3 times its K_m) was used for the α-191^{Lys} MoFe protein. The distribution of cis and trans isomers in the dideuterated product, $C_2D_2H_2$, was estimated by Fourier transform infrared spectroscopy. The result for each of the MoFe proteins is listed in Table 2 together with the propensity to produce C_2H_6 directly from C_2H_2. Both the wild-type and α-195^{Gln} MoFe proteins, which produce no C_2H_6 from C_2H_2 under regular assay conditions, produced very little (4% and 1%, respectively) trans isomer. The α-195^{Asn} and α-191^{Lys} MoFe proteins, which produce significant quantities (23% and 13% of total electron flux, respectively) of C_2H_6 from C_2H_2, also produced 37% and 21% *trans*-$C_2D_2H_2$, respectively. In this same experiment, these altered MoFe proteins allocated 22% and 11%, respectively, of electron flux to C_2H_6. The correlation coefficient relating ethane as a percentage of total electron flux to the percentage of *trans*-$C_2D_2H_2$ formed is 0.991. The production of C_2DH_3 was also measured by the intensity of the 943 cm^{-1} infrared band. It was found to account for less than 1% of the total hydrocarbon products, which is consistent with the starting concentration of C_2DH.

Ethylene as a Substrate and Inhibitor. The K_m for C_2H_4 reduction to C_2H_6 was determined for the wild-type and both histidine-195-substituted MoFe proteins, and the values are listed in Table 1. Table 3 shows that the three MoFe proteins, when complemented with Fe protein, reduced C_2H_4 to C_2H_6 at rates that varied from 11 to 75 nmol of C_2H_6 (min·mg of MoFe protein)$^{-1}$ under 50 kPa of C_2H_4 in argon (0.4−1 times

their K_m). Catalyzed C_2H_4 reduction accounted for only 0.5−6% of the total electron flux and was accompanied by a 17 ± 2% decrease in the overall rate of product formation. This slowing of the overall rate is accompanied by a similar decrease in the rate of MgATP hydrolysis as shown by the unchanged ATP/2e$^-$ ratio. Thus, MgATP hydrolysis remains tightly coupled to electron transfer to substrate. Similar observations have been made for the Mo-nitrogenases of both *Klebsiella pneumoniae* and *Azotobacter chroococcum* under 101 kPa of C_2H_4 (*5*). In contrast, the α-191^{Lys} MoFe protein did not reduce C_2H_4, but it did suffer a decrease, albeit smaller (9%), in total electron flux under 50 kPa of C_2H_4.

Pure *trans*-$C_2D_2H_2$ was used to determine whether Mo-nitrogenase, under turnover conditions, could scramble the label and produce a mixture of the *cis*- and *trans*-$C_2D_2H_2$ isomers. Neither the wild type nor the nitrogenase containing the α-195^{Asn} MoFe protein produced an infrared band at 843 cm^{-1} when turned over under 101 kPa of *trans*-$C_2D_2H_2$. These results indicate that neither enzyme is capable of producing the cis isomer when the trans isomer is being bound and reduced. In addition, no infrared band at 943 cm^{-1} arose, indicating that no label was lost and no C_2DH_3 formed. The intermediate, which is generated during C_2H_2 reduction and allows loss of stereospecificity during $C_2D_2H_2$ formation, must, therefore, be inaccessible to exogenous ethylene.

Carbon monoxide (CO; 10 kPa) inhibited catalyzed C_2H_4 reduction for the three MoFe proteins able to do so. However, the inhibition of electron flux was only partially relieved by CO, declining to 9 ± 4% with the wild-type and α-195^{Gln} and α-195^{Asn} MoFe proteins. The effect of CO on relieving electron-flux inhibition by C_2H_4 with the α-191^{Lys} MoFe protein is not amenable to simple analysis because CO also inhibits both H_2 evolution and total electron flux in this system and, in addition, uncouples MgATP hydrolysis from electron transfer to product. In the presence of 50 kPa of C_2H_4 and 10 kPa of CO, the total electron flux was decreased by 61%, which represents a 2.6-fold decrease in the rate of product (H_2) formation, and the ATP/2e$^-$ ratio was increased by 3.0-fold (Table 3). These changes in both the rate of product formation and ATP/2e$^-$ ratio are identical to those occurring under 10 kPa of CO alone in argon, suggesting that the inhibition by C_2H_4 may have been relieved by CO.

When 10 kPa of C_2H_2 was added instead of CO, the rate of C_2H_6 formation from C_2H_4 catalyzed by either the wild-type or the α-195^{Gln} MoFe protein decreased by 88 ± 3% of their C_2H_2-free rates. However, because nitrogenases containing either the α-195^{Asn} or the α-191^{Lys} MoFe protein produce C_2H_6 from C_2H_2, their C_2H_6-production rates increased. The α-195^{Asn} MoFe protein showed a 2.1-fold enhancement of the C_2H_6-production rate. Because the C_2H_4 produced from C_2H_2 reduction is indistinguishable from the substrate C_2H_4, neither total electron flux nor the ATP/2e$^-$ ratio can be calculated under these conditions. C_2H_6 production from C_2H_4 catalyzed by these nitrogenases was not inhibited under 50 kPa of C_2H_4/51 kPa of H_2 atmosphere. Added H_2 may, in fact, have stimulated C_2H_4 reduction (Table 3).

DISCUSSION

Various aspects of the reactivity of all four MoFe proteins used in this present work have been studied before. Under normal assay conditions, the α-195^{Gln} MoFe protein catalyzes the reduction of protons and HCN quite effectively when compared to the wild-type enzyme. However, unlike the wild type, C_2H_2 is a relatively poor substrate for this altered MoFe protein, which is also an ineffective catalyst for both dinitrogen and azide reduction. Furthermore, electron flux through the α-195^{Gln} MoFe protein is unaffected by CN$^-$ (*22*, *34*). In contrast, the previously reported specific activities of the α-195^{Asn} and α-191^{Lys} MoFe proteins toward protons and C_2H_2 are considerably lower than those of the wild type, and in addition to C_2H_4, C_2H_6 is a product of catalyzed C_2H_2 reduction by both (*21*, *28*). Our specific activities for these two altered MoFe proteins are in agreement with the previous measurements when calculated on a per-milligram-of-protein basis, but when adjusted for their measured Mo content, all specific activities become comparable to those of the wild type. Of related interest, the alternative nitrogenases, both V-nitrogenase (*40*, *41*) and Fe-nitrogenase (*42*), also catalyze the production of C_2H_6 from C_2H_2.

Interaction of Acetylene with the MoFe Protein. It was previously reported (*2*) that proton addition across the acetylenic triple bond of C_2D_2 was completely cis with wild-type *C. pasteurianum* nitrogenase. Other studies, using various wild-type nitrogenases, suggested the formation of trace amounts of *trans*-$C_2D_2H_2$ (*6*, *7*). Moreover, this high selectivity was used to argue in favor of a concerted transfer of two electrons and two protons to C_2D_2 (*43*). However, because of the high symmetry of C_2H_2, the validity of this suggestion has been questioned (*44*). A more recent study (*8*), using nitrogen-fixing *K. pneumoniae* cultures, reported that, depending on the culture conditions, 10% or more of the $C_2D_2H_2$ product was the trans isomer.

To gain further insight into the interaction of C_2H_2 with nitrogenase, we measured the proportions of both $C_2D_2H_2$ isomers produced by each of the four MoFe proteins. These data were then used to determine whether a correlation existed between the stereospecificity of proton addition and the production of ethane. The affinity of wild-type nitrogenase for C_2H_2 is much greater than its affinity for C_2H_4 (*5*; this work). Therefore, as soon as C_2H_4 is formed, it would be displaced from the active site by C_2H_2, and so no C_2H_6 is formed (*5*, *28*, *41*). However, if ethane is formed, it is likely that the substrate languishes for an extended period of time on the enzyme, indicating a lower affinity for C_2H_2 and/or a higher affinity for C_2H_4. In these circumstances, any enzyme-bound intermediate should then have time to rearrange and so lose the stereochemical specificity of its reduction and protonation. It might have been expected that the percentage of electron flux going to H_2 evolution would also reflect the affinity for C_2H_2. However, although the wild-type and α-195^{Gln} MoFe proteins have the same K_m (0.5 kPa) for C_2H_4 formation from C_2H_2, they produce H_2 at substantially different rates under 10 kPa of C_2H_2.

Both the wild-type and α-195^{Gln} MoFe proteins have a low K_m for C_2H_4 formation from C_2H_2, and neither produces C_2H_6 from C_2H_2. These observations are consistent with the suggestion that too high a C_2H_2 affinity results in a loss of C_2H_6-production potential. In contrast, both the α-195^{Asn} and α-191^{Lys} MoFe proteins have a higher K_m for C_2H_2 reduction, and both produce C_2H_6. The α-191^{Lys} MoFe protein has the highest K_m (35 kPa) for C_2H_4 formation, and as expected, it produces very little C_2H_6 under 10 kPa of C_2H_2. These data

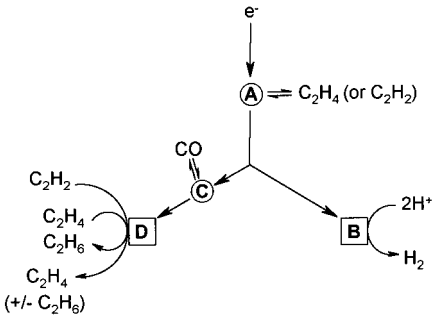
suggest that the C_2H_2 affinity of the α-191^{Lys} MoFe protein is only just sufficient to allow effective C_2H_2 binding. These observations are consistent with our predicted consequence of very low C_2H_2 affinity, which is that C_2H_6 production will be marginal. Because of this high K_m for C_2H_4 production from C_2H_2 for the α-191^{Lys} MoFe protein, electron distribution to products was determined at 101 kPa of C_2H_2 (3 times its K_m) to give a more meaningful comparison to the other MoFe proteins. Under this condition, the α-191^{Lys} MoFe protein directed 58% of the total electron flux to produce C_2H_4 and C_2H_6, an amount similar to that used by the other altered MoFe proteins. With the α-195^{Asn} MoFe protein, the appropriate C_2H_2 affinity for C_2H_6 production appears to have been achieved at its K_m of 1 kPa. These data suggest that the affinity for C_2H_2 is the major factor in producing C_2H_6 from C_2H_2, which is achieved through a delicate balance between having an affinity for C_2H_2 that is high enough to have reasonable binding but not so high that it effectively displaces the bound intermediates. A similar argument may apply to the V-nitrogenase of *A. chroococcum* where the K_m for C_2H_2 reduction (7 kPa) is also higher than for wild-type Mo-nitrogenase and C_2H_6 is an appreciable product of C_2H_2 reduction (*41*).

Even though only two of the four MoFe proteins produced C_2H_6, all four produced some *trans*-$C_2D_2H_2$ from C_2D_2. By far, the most extensive loss of stereospecificity occurred with the α-195^{Asn} and α-191^{Lys} MoFe proteins. Here, the percentage of *trans*-$C_2D_2H_2$ was 5−9 times greater than with the others. These altered MoFe proteins are the same ones that exhibited C_2H_6 production from C_2H_2. These data are clearly correlated and are consistent with the hypothesis outlined above that a common, longer-lived, ethylenic intermediate on the enzyme is responsible for both ethane production and loss of proton-addition stereospecificity.

Ethylene as a Substrate. Because of the likely intermediacy of an ethylenic derivative during catalyzed C_2H_2 reduction, we next chose to study directly the catalyzed reduction of C_2H_4. We predicted that the altered nitrogenase, which produced the most C_2H_6 product from C_2H_2 substrate, would produce the most C_2H_6 product from C_2H_4 substrate. Ethylene has been shown previously to bind to the FeMo-cofactor of the nitrogenase MoFe protein (*45*) and to be a poor substrate for the wild-type enzymes from both *K. pneumoniae* and *A. chroococcum* (*5*). This observation also held for the *A. vinelandii* nitrogenases studied here (see Table 3). With the exception of the nitrogenase containing the α-195^{Asn} MoFe protein, the *A. vinelandii* enzymes had very low specific activities for C_2H_4 reduction and delivered less than 1% of their total electron flux to C_2H_6 formation. However, consistent with our prediction, the α-195^{Asn} MoFe protein had a specific activity for C_2H_6 formation 10-fold higher than the others. These results further suggest that the C_2H_4-reduction site is affected by substitution at residue α-195, although whether the effect(s) are related to the substrate-reduction site directly or are mediated through interruption of the delivery of electrons/protons is unclear.

Ethylene inhibited total electron flux to the same extent for all four *A. vinelandii* nitrogenases without uncoupling MgATP hydrolysis from electron transfer. This observation indicates that the C_2H_4 binding flux-inhibition site is remotely located from residues α-191 and α-195 and is unaffected by substitutions at these positions. As expected for a Mo-nitrogenase substrate and just as with the *K. pneumoniae* nitrogenase (*5*), added CO eliminated C_2H_4 reduction, but it did not fully relieve the electron-flux inhibition for any of the four *A. vinelandii* nitrogenases. This observation indicates that electron-flux inhibition is not directly connected to C_2H_4 reduction. This situation suggests that C_2H_4 has two binding sites and the presence of CO affects only its reduction site. If so, then either two C_2H_4 molecules or one molecule each of CO and C_2H_4 can bind simultaneously (see Scheme 1).

Scheme 1: Likely Arrangement of Substrate and Inhibitor Binding Sites along the Electron-Transfer Pathway[a]



[a] Evidence exists that sites B, C, and D are located either on or close to the FeMo-cofactor (*19*−*22*, *34*, *45*, *55*). Two C_2H_2 binding sites on Mo-nitrogenase have been recognized (*4*, *56*), a high-affinity and a low-affinity site. Site D is the high-affinity C_2H_2 binding site, which is susceptible to inhibition by CO. Site A may represent the low-affinity C_2H_2 binding site, which contributes little toward C_2H_2 reduction under normal assay conditions, but when occupied by either C_2H_2 or C_2H_4, under high-substrate-concentration conditions, may cause inhibition of total electron flux. Because it escapes the effects of bound CO (see the text), site A may be located elsewhere on the MoFe protein, possibly on the P cluster.

Under a mixed C_2H_4/C_2H_2/Ar atmosphere, the C_2H_4-reduction rate was inhibited by about 90% for nitrogenases containing either the wild-type or the α-195^{Gln} MoFe protein. This result is consistent with their similar and much lower K_m for C_2H_2 compared to C_2H_4. In fact, one might have expected that C_2H_4 reduction would have been completely abolished. A similar analysis of the effect of added C_2H_2 on catalyzed C_2H_4 reduction by the nitrogenases, which contain either the α-195^{Asn} or the α-191^{Lys} MoFe protein, is complicated by the fact that both reduce C_2H_2 directly to C_2H_6. With the α-195^{Asn} MoFe protein, an increase (2-fold) in the C_2H_6-formation rate occurred as expected because the α-195^{Asn} MoFe protein has the highest specific activity for C_2H_6 production directly from C_2H_2. Of course, it is difficult to measure directly total electron flux under this mixed C_2H_4/C_2H_2/Ar atmosphere because the C_2H_4 substrate masks the C_2H_4 produced from C_2H_2 reduction. However, if it is assumed that all C_2H_6 produced was from the four-electron reduction of added C_2H_2, then it is possible to calculate an overall rate of product formation on the basis of the known ratio of hydrocarbon products for the α-195^{Asn} MoFe protein (see Table 1). Taken together with the H_2-evolution rate, the calculated rate of product formation would be 1262 nmol of electron pairs $(min \cdot mg)^{-1}$ compared to 1467 nmol of electron pairs $(min \cdot mg)^{-1}$ under 101 kPa of Ar. Of this calculated rate, the combined rates of C_2H_4 and C_2H_6 production would account for 74% compared to the normal 69% (Table 1). Therefore, it is likely that C_2H_2 completely eliminates C_2H_6 production from C_2H_4 but does not relieve electron-flux inhibition. Making the same assumption for the nitrogenase

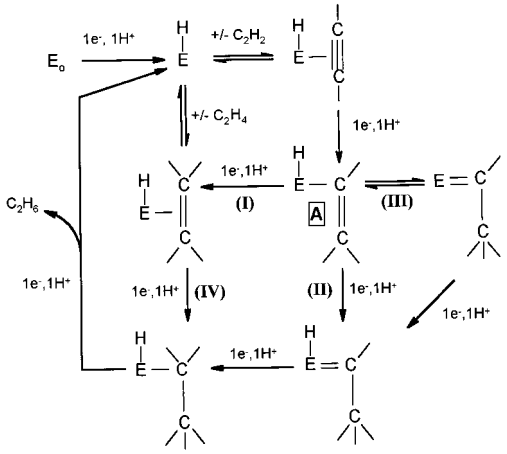
containing the α-191^{Lys} MoFe protein produces the same conclusion. The calculated rate of total product formation is 1352 nmol of electron pairs (min·mg)$^{-1}$, which is similar to the C_2H_4-inhibited rate and significantly lower than the rate of 1532 nmol (min·mg)$^{-1}$ under 101 kPa of Ar. These data suggest that C_2H_2 and C_2H_4 are reduced at the same site on the MoFe protein as shown in Scheme 1.

Some insight into the origin of the electron-flux inhibition by C_2H_4 comes from the observation that the rates of product formation and MgATP hydrolysis are simultaneously and equally diminished by C_2H_4. Thus, C_2H_4 cannot be only interrupting intermolecular electron transfer between the Fe protein and MoFe protein because this situation would lead to an elevated ATP/2e$^-$ ratio due to the requirement for MgATP hydrolysis for dissociation of the complex whether or not an electron is transferred (*46*). It is possible that bound C_2H_4 exerts its effect similarly to that of bound C_2H_2, which inhibits total electron flux by enhancing the MoFe protein−Fe protein association rate and so increasing the steady-state concentration of the inhibitory complex of the MoFe protein with oxidized Fe protein (*47*). This interpretation requires that this ternary complex does not catalyze reductant-independent MgATP hydrolysis, which is a property of the binary complex of MoFe protein with oxidized Fe protein in the absence of C_2H_2 (*48*); otherwise an elevated ATP/2e$^-$ ratio would result. Alternatively, bound C_2H_4 could prevent association of the two proteins to form the catalytically active complex. In this situation, and consistent with our observations, neither electron transfer nor MgATP hydrolysis could occur while C_2H_4 was bound to its electron-flux-inhibition site.

The inhibition of electron flux by C_2H_4 must be inherently different from the electron-flux inhibition caused by CN$^-$ (*49*). This contention has its basis in the following observations. First, with wild-type nitrogenase, electron-flux inhibition by CN$^-$ uncouples MgATP hydrolysis from electron transfer whereas, with C_2H_4 inhibition of flux, they remain tightly coupled. Further, the extent of electron-flux inhibition by CN$^-$ is much greater than with C_2H_4 (about 60% vs 16%). This difference is further highlighted by our observation that, unlike C_2H_4, which inhibited electron flux through all four MoFe proteins, CN$^-$ had little or no effect on the α-195^{Gln} MoFe protein (*34*). Second, CO relieves fully the electron-flux inhibition by CN$^-$ with wild-type nitrogenase but only partially relieves flux inhibition caused by C_2H_4. However, CO's relief of electron-flux inhibition by CN$^-$ cannot be due to them sharing a common binding site as has been suggested (*49*) because all four MoFe proteins bind CO whereas the α-195^{Gln} MoFe protein does not apparently bind CN$^-$. Together, these data indicate that C_2H_4 inhibits electron flux by binding to a site separate from the site that binds CN$^-$ and that these two sites are distinct from the site that binds CO.

Scheme 2 attempts to correlate what is known about the catalyzed reduction of both C_2H_2 and C_2H_4, including the stereospecific protonation. The key *σ*-alkenyl intermediate (labeled A) in the scheme has the following options. It can (a) accept one more electron and one more proton to become bound C_2H_4 if the proton is transferred to its α-carbon (route I), (b) accept one more electron and one more proton to become bound =CH−CH$_3$ if the proton is transferred to its *β*-carbon (route II), or (c) reversibly form bound =CH−CH$_3$ without additional electron/proton input (route III). However, after the third electron is accepted, the enzyme is committed to form product. Route I would result in C_2H_4 production from C_2H_2, route II would produce C_2H_6 from C_2H_2, and route III would account for any loss of stereospecific protonation of the C_2H_4 released because free rotation would now be possible around the C−C single bond of the bound =CH−CH$_3$ group. Route IV allows the production of C_2H_6 from C_2H_4.

Scheme 2: Postulated Mechanisms for C_2H_2 Reduction to C_2H_4 plus C_2H_6 and C_2H_4 Reduction to C_2H_6 Catalyzed by Mo-Nitrogenases[a]



[a] All protons attached to C atoms are omitted for clarity. The high-affinity substrate binding site represented by E resides on the FeMo-cofactor prosthetic group of the MoFe protein (*20*, *21*).

Scheme 2 is consistent with the suggested action of nitrogenase and the known chemistry of model compounds. First, C_2H_2 does not bind to the as-isolated MoFe protein but does so only after the MoFe protein has accepted either one or two electrons (*47*). For simplicity, the scheme only considers C_2H_2 binding to the MoFe protein after one electron has been accepted. Further, it conforms to the suggestion that C_2H_4 is released only after three electrons have been accepted. Second, the reversible binding of C_2H_4 at the one-electron-reduced level of the MoFe protein is consistent with the inhibition by C_2H_2 of C_2H_4 reduction (*5*; this work). Third, the scheme provides a common pathway for the production of both C_2H_4 and C_2H_6 directly from C_2H_2 reduction (*28*). Fourth, interconversion of *cis*- and *trans*-$C_2D_2H_2$ isomers is not allowed and is not observed when exogenous $C_2D_2H_2$ is supplied. Fifth, both the key *σ*-alkenyl intermediate and its *β*-elimination chemistry are consistent with model studies (*50*−*53*).

Applying Scheme 2 to our data, nitrogenases incorporating either the wild-type or α-195^{Gln} MoFe protein must use route I almost exclusively because neither produces any C_2H_6 from C_2H_2 and both are effectively stereospecific in their protonation of C_2H_2. Some use of route IV is apparent because some C_2H_6 is produced from C_2H_4. In contrast, the nitrogenase using the α-191^{Lys} MoFe protein can apparently make use of routes I−III because it produces C_2H_4 and C_2H_6 from C_2H_2 and also suffers significant loss of protonation stereospecificity, but it cannot use route IV because no C_2H_6 is produced from C_2H_4. Perfectly poised to take full advantage of all situations is the nitrogenase with the α-195^{Asn} MoFe protein. It produces significant quantities of

C_2H_6 from both C_2H_2 and C_2H_4 and also loses considerable stereospecificity in the protonation to give C_2H_4. It must, therefore, be capable of using all available (I−IV) routes.

It has been pointed out that, although the V-nitrogenase from *A. chroococcum* catalyzes the production of both C_2H_4 and C_2H_6 from C_2H_2, its mechanism of doing so is significantly different from that operating in the altered Mo-nitrogenases (*28*, *41*). The V-nitrogenase also is very stereospecific in its protonation of C_2H_2 (*41*). This combination of properties is not observed with any of our MoFe proteins, where retention of stereospecificity of protonation is never accompanied by the ability to produce C_2H_6 from C_2H_2. This relationship is another significant mechanistic difference between the V- and Mo-nitrogenases and their interactions with alkynes and alkenes. Moreover, unlike the Mo-nitrogenases, V-nitrogenase shows a lag before C_2H_6 formation from C_2H_2 is observed, but no lag is observed with C_2H_6 formation from C_2H_4. Therefore, for V-nitrogenase, these processes cannot involve the same intermediate as indicated in the above scheme for the Mo-nitrogenase.

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