Effects on Substrate Reduction of Substitution of Histidine-195 by Glutamine in the α-Subunit of the MoFe Protein of *Azotobacter vinelandii* Nitrogenase†

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ABSTRACT: Studies of the substrate-reducing capabilities of an altered nitrogenase MoFe protein (α-195^{Gln} instead of α-195^{His}) from a mutant of *Azotobacter vinelandii* show, contrary to an earlier report [Kim, C.-H., Newton, W. E., and Dean, D. R. (1995) *Biochemistry 34*, 2798−2808], that the α-195^{Gln} MoFe protein can reduce N_2 to NH_3 but at a rate that is <2% of that of the wild type. The extent of effective binding of N_2 by this altered MoFe protein, as monitored by the inhibition of H_2 evolution, is markedly increased as temperature is lowered but virtually eliminated at 45 °C. This inhibition of H_2 evolution results in an increase in the ATP:2e$^-$ ratio, i.e., the number of molecules of MgATP hydrolyzed for each electron pair transferred to substrate, from ca. 5 (the wild-type level) at 45 °C to nearly 25 at 13 °C. Like wild-type nitrogenase, the N_2 inhibition of H_2 evolution reaches a maximum at an Fe protein:MoFe protein molar ratio of ca. 2.5, suggesting that a highly reduced enzyme may not be necessary for N_2 binding. N_2 binding to the α-195^{Gln} MoFe protein retains a hallmark of the wild type by producing HD under a mixed N_2/D_2 atmosphere. The rate of HD production and the fraction of total electron flow allocated to HD are similar to those for wild-type nitrogenase under the same conditions. However, the electrons forming HD do not come from those normally producing NH_3 (as occurs in the wild type) but are equivalent to those whose evolution as H_2 had been inhibited by N_2. N_2 also inhibits C_2H_2 reduction catalyzed by the α-195^{Gln} nitrogenase. This inhibition is relieved by added H_2, resulting in a lowering of the elevated ATP:2e$^-$ ratio to that found under Ar. With solutions of NaCN, which contain both the substrate, HCN, and the inhibitor, CN^-, reduction of HCN is not impaired with the α-195^{Gln} nitrogenase, but the inhibition by CN^- of total electron flow to substrate, which is observed with the wild-type MoFe protein, is completely absent. Unlike that of the catalyzed reduction of H^+, HCN, or C_2H_2, the extent of azide reduction to either N_2 or N_2H_4 is markedly decreased (to 5−7% of that of the wild type) with the α-195^{Gln} nitrogenase. Azide, like N_2, inhibits H_2 evolution and increases the ATP:2e$^-$ ratio. Both effects are freely reversible and abolished by CO. Added D_2 does not relieve either effect, implying that N_2 produced from N_3^- is not the inhibitory species. The correlation between the extremely low rates of reduction for both N_2 and azide by the α-195^{Gln} nitrogenase and their common ability to inhibit H_2 evolution suggests that α-histidine-195 may be an important proton conductor to the FeMo cofactor center and specifically required for reduction of these two substrates.

Nitrogen fixation in biological systems is catalyzed by nitrogenase, the most common form of which is called Mo-nitrogenase which consists of two metal-centered proteins termed the Fe protein and the MoFe protein. It is currently believed that, during catalyzed substrate reduction, the Fe protein delivers electrons one at a time to the MoFe protein in a sequence involving protein−protein association and dissociation accompanied by hydrolysis of a minimum of two molecules of MgATP per electron transfer.

The three-dimensional structures of the two component proteins of Mo-nitrogenase, which have been described in a steady stream of publications over the last five years (*2*−*11*), have led to advances in our understanding of their biochemical function (see refs *12* and *13* for reviews). In particular, the structure, positioning, and bonding of a molybdenum- and iron-containing cluster, called the FeMo cofactor, within the MoFe protein have been defined. Because the evidence is compelling that the FeMo cofactor is the site of substrate binding and reduction (*1*, *14*−*17*), defining its environment should allow a detailed understanding of which substrates bind where on the FeMo cofactor.

On the basis of amino acid sequence conservation among MoFe proteins from many different bacterial genera and species, certain residues in the protein were suggested (*17*−*23*) to either bond directly to the FeMo cofactor or be close enough to the FeMo cofactor to influence its electronic environment and, thereby, modify substrate binding or reduction. One such residue in the α-subunit is histidine-195 which, prior to the crystallographic determination, was considered a possible covalent ligand to the FeMo cofactor

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(*24*). An altered MoFe protein in which this residue was replaced by asparagine showed significantly different catalytic and spectroscopic properties compared to the wild type (*16*, *17*) and was incapable of interacting with N_2. The crystal structure, however, shows that α-histidine-195 is not covalently linked to the FeMo cofactor, but is close enough to it (3.2 Å; see ref *12*) to form a hydrogen bond to one of the sulfides of the FeMo cofactor. Replacement of this residue by a number of other amino acids (Gln, Tyr, Leu, Thr, or Gly) results in altered MoFe proteins with a variety of phenotypes. None of these altered MoFe proteins has been reported to be able to reduce N_2 (*1*), but all retain a range of activity for proton reduction to H_2 and C_2H_2 reduction either to C_2H_4 alone or to a mixture of C_2H_4 and C_2H_6. Analysis of the catalytic and spectroscopic properties of these altered MoFe proteins suggests that the α-histidine-195 residue helps to correctly position the FeMo cofactor within the protein so that N_2 binding can occur (*1*).

The MoFe protein in which the α-histidine-195 has been replaced with glutamine (abbreviated α-195^{Gln} MoFe protein) is one of the most interesting. The nitrogenase containing this altered MoFe protein still interacts with N_2, as evidenced by the fact that N_2 inhibits H_2 evolution and C_2H_2 reduction, both of which otherwise occur at rates similar to those for the wild-type MoFe protein (*1*). This inhibition by N_2 of H_2 evolution is not accompanied by an inhibition of ATP hydrolysis. Thus, a significant increase occurs in the ATP:$2e^-$ ratio, which is a measure of how many molecules of MgATP are hydrolyzed for each pair of electrons transferred to substrate (*1*). The α-195^{Gln} MoFe protein is also somewhat more sensitive to CO inhibition of both N_2 binding and C_2H_2 reduction (*1*). Despite the fact that the interaction of the nitrogenase containing the α-195^{Gln} MoFe protein (α-195^{Gln} nitrogenase) with N_2 was demonstrated by these indirect means, it was reported (*1*) that (i) N_2 could not be reduced to NH_3 and (ii) the enzyme-bound intermediate, which is observed for the wild type and which produces N_2H_4 on acid quenching (*25*), could not be detected.

Because of its high H^+ and C_2H_2 reduction rates, we initially sought to characterize the ability of the α-195^{Gln} nitrogenase to catalyze the reduction of other alternative substrates, namely, azide and cyanide. Solutions of sodium cyanide contain both HCN and CN^-, the relative amounts of each depending on the pH. The actual nitrogenase substrate is HCN. It is reduced by wild-type nitrogenase both by six electrons to yield CH_4 and NH_3 and by four electrons to produce CH_3NH_2 (methylamine) (*26*). In contrast, the anion, CN^-, acts not as a substrate but as a potent inhibitor of the electron flow to substrate, increasing the ATP:$2e^-$ ratio. CO and, to some extent, azide can reverse this inhibition by CN^- (*26*).

Azide ion was, at one time, regarded as a unique anionic nitrogenase substrate, but others are now known (see refs *27* and *28* for reviews). Like sodium cyanide solutions, sodium azide provides two species in solution, but unlike sodium cyanide, both species can be reduced by Mo-nitrogenase. Catalyzed reduction by Mo-nitrogenase (*29*−*34*) results in three major products, namely, N_2, N_2H_4, and NH_3. The two-electron reduction of N_3^- results in a 1:1 molar ratio of N_2:NH_3, while the six-electron reduction of HN_3 leads to a 1:1 molar ratio of N_2H_4:NH_3. The K_m for HN_3 for this latter reaction is the lowest for any nitrogenase substrate (*34*). However, "excess" NH_3 is always observed, i.e., more NH_3 than can be accounted for by the sum of N_2 and N_2H_4 produced. This result has been interpreted as the N_2 produced from N_3^- reduction acting as a substrate and being further reduced to ammonia. This interpretation is supported by the observation that added D_2, which inhibits only the reduction of N_2 among all nitrogenase substrates, results in a decrease in the level of NH_3 production and an increase in the level of N_2 production (*34*). It should be noted, however, that even 101 kPa D_2 did not eliminate "excess" ammonia production, which might have been expected in view of the extremely low N_2 concentrations in the assays.

The location, fate, and effects of the N_2 produced from N_3^- reduction are of interest. It was reported (*34*) that it could not be in equilibrium with free N_2 because the amount of N_2 reduced was very much greater than the amount that could have been reduced with exogenously added N_2 at the calculated concentration. However, it was noted (*33*) that $^{15}N_2$ in the gas phase over nitrogenase, which was reducing "azide", resulted in $^{15}NH_3$, implying that N_2 from N_3^- does not have unique access to the enzymatic site that is reducing N_2. These conflicting results with wild-type nitrogenase led us to reconsider the interaction of N_2 with the α-195^{Gln} nitrogenase and specifically to determine any effect of H_2 (or D_2) on the inhibition by N_2 of electron flow to substrate exhibited by nitrogenase containing this altered MoFe protein.

Dihydrogen (H_2) involvement with nitrogenase catalysis is particularly complex. Not only is H_2 a product of nitrogenase turnover, but it is also a competitive inhibitor of N_2 reduction to NH_3 (*35*, *36*). N_2 is the only nitrogenase substrate whose reduction is inhibited by H_2 (*32*, *36*, *37*). Another feature of H_2−N_2 interactions is the nitrogenase-catalyzed formation of HD in the presence of N_2 and D_2 (*37*−*42*). HD formation is dependent on MgATP, reductant, and N_2 and is not a simple D_2−H_2O exchange process because (i) incubations under tritium-labeled H_2 gas produce insignificant radioactivity in the liquid phase (*37*) and (ii) incubation of *Azotobacter vinelandii* nitrogenase under N_2 and HD does not produce D_2 (*43*). Electrons appearing as HD under N_2/D_2 (one electron is required for each HD formed; *37*, *44*) are diverted exclusively from NH_3 formation, and therefore, concomitant H_2 evolution is unaffected. The $K_m(N_2)$ for HD formation is considerably lower than the K_m-(N_2) for NH_3 production (*37*, *41*, *45*). Because of the N_2 dependence of HD formation, HD production and the inhibition of the N_2-to-NH_3 reaction by H_2 have been considered different manifestations of the same nitrogenase chemistry, and a variety of models have been proposed to incorporate both phenomena.

The first model suggested that HD was formed by the reversible exchange of D_2 with an enzyme-bound nitrogenous intermediate (*38*). It implied the occurrence of a two-electron reduction of N_2 to produce a reactive, enzyme-bound, diazene level (N_2H_2) intermediate, which was decomposed by reaction with a D_2 molecule to produce two HD molecules and release the N_2 intact. This reaction would account for the stoichiometry of one electron per HD and the observation that a small amount of N_2 is capable of producing a considerable quantity of HD (*37*). D_2, therefore, is proposed to inhibit N_2 reduction by intercepting a partially reduced and partially protonated N−N species normally involved in

N_2 reduction to NH_3 and then releasing N_2. The model has the shortcoming that interception of the enzyme-bound intermediate by D_2 (or H_2) would predict noncompetitive inhibition by H_2 of N_2 reduction rather than the actual competitive pattern found experimentally (*40*).

The second model (*40*) differs in suggesting that both N_2 and D_2 bind to the same form and/or state of nitrogenase without N_2 reduction being required. In the absence of prebound D_2, N_2 binds and is reduced to NH_3. However, when D_2 is bound before N_2, N_2 reduction is aborted and HD results. To explain the lower $K_m(N_2)$ for HD formation compared with the $K_m(N_2)$ for NH_3 production, N_2 is postulated to promote HD formation by binding to enzyme carrying D_2. However, because the model also predicts that high N_2 pressure will completely inhibit HD formation by competing with D_2 for the enzyme, a delicate balance between N_2 stimulation and N_2 inhibition of HD formation must exist. This model also predicts that the minimum percentage of electrons (25%) going to H_2 evolution during N_2 reduction (*46*, *47*) should be maintained at high pD_2 where no NH_3 should be produced. With hyperbaric assay procedures, this prediction can readily be tested.

In a third model attributed to Cleland (*40*), N_2 normally binds by displacement of H_2, but when D_2 displaces N_2, the deuterated enzyme produces HD. This model implies a 1:2 ratio between H_2 and HD production; i.e., each product accounts for 50% of the total electron flow. An increase in pD_2 should, therefore, enhance H_2 evolution through its inhibition of N_2 reduction and lower the percentage of electron flux going to HD and NH_3. Most data sets show no such increase in H_2 evolution (*36*, *40*, *48*), but one report (*42*) notes an enhancement by D_2 of H_2 evolution at the expense of HD and NH_3 and an extrapolation of the 2HD:H_2 ratio to 1 at saturating N_2 levels and an infinite pD_2, supporting the third model.

The Thorneley−Lowe model for nitrogenase action explains HD formation by requiring the production of a form of the enzyme reduced by three electrons, two involved in a dihydride and one elsewhere, but with a separately bound H^+ [E_3−$H_2(H^+)$] (*13*, *49*). N_2 binds to this intermediate by displacing H_2, but N_2 can, in its turn, be displaced by D_2 to produce E_3−$D_2(H^+)$. Internal electron transfer then results in HD and E_1D which, after addition of two further solvent protons and two electrons, gives a further HD and E_1H. An important restriction of this model is that the proton (shown in parentheses) cannot exchange with species generated from gaseous H_2, HD, or D_2 (*43*). The Cleland model is in fact an abbreviated version of the Lowe−Thorneley scheme that does not consider the possibility that the evolution of two HD molecules might involve two different reaction mechanisms with different rate constants.

The α-195^{Gln} MoFe protein offers an opportunity to test some aspects of these models because it is capable of binding N_2 (*1*). If no reduced nitrogenous intermediates can be produced by this altered MoFe protein, then HD formation by the α-195^{Gln} MoFe protein would discriminate against the diazene mechanism. Here, we also show that the α-195^{Gln} MoFe protein functions very differently with respect to cyanide and azide reduction compared to the wild type, and the results raise important questions about the coupling of electron transport to ATP hydrolysis in nitrogenase.

EXPERIMENTAL PROCEDURES

Cell Growth and Protein Purification. Wild-type (α-195^{His}) and DJ540 (α-195^{Gln}) strains of *A. vinelandii* were grown in a 24 L fermenter at 30 °C in a modified, liquid Burk medium (*35*). Nitrogenase derepression and cell extract preparation were performed as previously described (*17*). Cell extracts were heat treated for 5 min at 50 °C and cooled before centrifuging 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. The wild-type and altered MoFe proteins were further purified by gel filtration before phenyl-Sepharose hydrophobic interaction chromatography was carried out (*1*). The purified Fe protein and wild-type and α-195^{Gln} MoFe proteins had specific activities of 2800, 3000, and 3200 nmol of H_2 produced min^{-1} mg^{-1}, respectively. Protein concentrations were determined by the method of Lowry (*50*). SDS−PAGE with Coomassie Blue staining was used to confirm that all proteins were homogeneous (*51*). 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) was carried out by dialysis in an anaerobic glovebox with a dioxygen concentration of less than 1 ppm. Unless stated, all buffers were saturated with argon and contained 2 mM sodium dithionite.

Preparation of Triply ^{15}N-Labeled Azide. Potassium azide labeled with ^{15}N in all three nitrogen atoms was synthesized by a modification of the published method (*52*). Hydrazine sulfate (1 g, >98% ^{15}N, Cambridge Isotope Laboratories, Andover, MA) was stirred for 72 h with a solution containing 0.97 g of KOH in 5 mL of methanol in a 40 mL reaction vial sealed under argon to prevent oxidation of hydrazine. The slurry of K_2SO_4 was filtered off through a sintered funnel and washed with sufficient methanol to recover 5 mL of solubilized hydrazine in methanol; the hydrazine recovery was 88%. This solution was added to a second solution produced by dissolving 0.85 g of potassium metal in 5.4 mL of cold methanol and adding 10.8 mL of cold diethyl ether.

Ethyl nitrite was produced by treating dropwise with stirring 1 g of $Na^{15}NO_2$ (>98% ^{15}N, Cambridge Isotope Laboratories), which was dissolved in 2 mL of cold water, with a cold mixture of 1.74 mL of ethanol and 1.24 mL of concentrated HCl. It was distilled in a cold room through a 10 cm condenser cooled with ice/water directly into the hydrazine solution. The potassium azide was recovered as described previously (*52*); the yield was 0.45 g (82% on the basis of extracted hydrazine).

Nitrogense Assays. All activities of the wild-type and α-195^{Gln} MoFe proteins were measured with a 20-fold molar ratio of wild-type Fe protein, unless otherwise stated. Assays were conducted in 9.25 mL reaction vials fitted with butyl rubber stoppers 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. Reactions were terminated by injection of 0.3 mL of 0.5 M EDTA (pH 7.5).

Assays under hyperbaric pN_2 were performed in the same vials containing 0.05 mL of 10% (v/v) C_2H_4 as a marker gas and injected with the appropriate additional volume of

N_2 immediately after protein addition. Such assays were vented to ambient pressure before gas samples were analyzed for H_2; the proportion of gas lost was assessed by measuring the recovery of C_2H_4 and correcting the recovered H_2 accordingly.

Gaseous products were measured by gas chromatography. For evolved dihydrogen, a molecular sieve 5A column (Supelco, Bellefonte, PA) and a TCD detector were used, whereas methane and ethylene were quantified with a Porapak N column and a FID detector.

Hydrazine was measured spectrophotometrically with *p*-(dimethylamino)benzaldehyde (*33*). After the contents of each vial were treated by passage through a 2.5 cm × 6 mm column of Dowex-1X2 (Cl^-) (*53*), creatine content (as an index of ATP hydrolysis) was measured by the method of Ennor (*54*) and ammonia content by the indophenol method (*53*). Because the extent of ammonia production from azide is very small despite extensive ATP hydrolysis, creatine interference with the indophenol method cannot be adequately corrected. In such cases, the effluent from the Dowex-1X2 column was applied to a 1.5 cm × 6 mm column of Dowex-50X12 (Na^+ form). After two 0.5 mL washes with water and two with 0.25 M NaCl, all creatine had passed through the column, and ammonia could then be eluted quantitatively with four 0.5 mL aliquots of 2 M NaCl (*55*). Elution with NaCl is preferable to that with acid because the indophenol method is unaffected by 2 mmol of NaCl added to the 2.7 mL assay, whereas careful neutralization is required for acidified samples.

Dinitrogen produced from $K^{15}N_3$ was measured as the mass 30 peak on a model 7070 E-HF mass spectrometer (VG Analytical, Manchester, U.K.) and quantified by reference to the argon background after determining the relative ionization efficiencies for N_2 and Ar. The amounts of dihydrogen (H_2) and HD produced in incubations under deuterium (D_2) were also measured either mass spectrometrically using an added internal argon standard or directly with reference to D_2 after determination of the relative ionization efficiencies for H_2, D_2, and Ar. For HD, an ionization efficiency intermediate between those of H_2 and D_2 was assumed.

Preparation of a NaCN Stock Solution for Nitrogenase Assays. A 100 mM NaCN stock solution was prepared anaerobically by flushing the desired amount of solid NaCN in a sealed serum vial prior to addition of degassed 25 mM HEPES (pH 7.4). A predetermined amount of degassed 9 M HCl was injected into the vial to obtain the desired pH. Appropriate aliquots of the stock solution were added by syringe to each anaerobic reaction vial during the temperature incubation period. NH_3, CH_4, and H_2 contents were determined from all assays containing NaCN; however, the formula, 2 × nanomoles of H_2 + 7.88 × nanomoles of CH_4 (*26*), was used to calculate the total number of electron pairs going to the product to compensate for CH_3NH_2 production, which was not determined.

RESULTS

Azide Reduction. To avoid confusion, we use either N_3^- or azide ion to refer to the charged species, either HN_3 or hydrazoic acid to refer to the uncharged molecule, and "azide" to describe collectively all species in solutions of $Na(K)N_3$. Table 1 shows that N_2H_4 and N_2 are both products of "azide" reduction with the α-195^{Gln} MoFe protein, as with the wild type (*33*, *34*), but the rate of reaction with the α-195^{Gln} MoFe protein is only 7.5% of that of the wild type for N_3^- reduction to N_2 and 5.5% of that for HN_3 reduction to N_2H_4. Both wild-type and α-195^{Gln} MoFe proteins also catalyze excess ammonia production (ammonia formed over and above the sum of N_2 and N_2H_4; *33*, *34*). Although the rate for this last reaction is small, it is consistently observed. Excess ammonia production by the α-195^{Gln} MoFe protein is not inhibited by 101 kPa H_2.

Table 1: Specific Activities [nmol min^{-1} (mg of MoFe protein)$^{-1}$] for Proton and "Azide" Reduction by Wild-Type and α-195^{Gln} MoFe Proteins

	argon		argon and 10 mM NaN_3				
protein	H_2	ATP:2e$^-$	H_2	N_2	N_2H_4	NH_3	ATP:2e$^-$
wild-type	2604	4.5	1291	627	163	876	5.3
α-195^{Gln}	2711	4.8	752	47	9	83	23.7

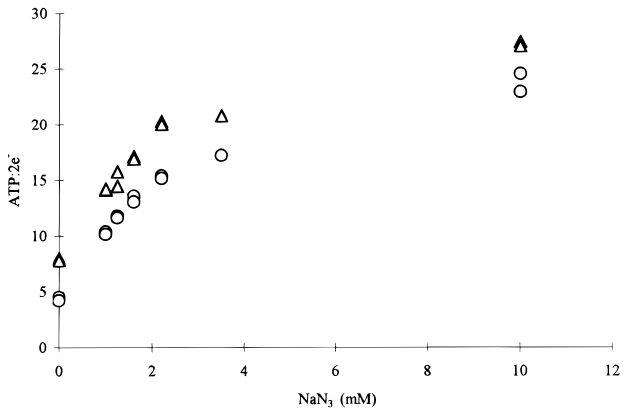

FIGURE 1: ATP:2e$^-$ ratio for assays of the α-195^{Gln} MoFe protein under N_2 (△) or Ar (○) in the presence of varying concentrations of sodium azide.

Table 2: Relief of "Azide" Inhibition of H_2 Evolution [nmol min^{-1} (mg of protein)$^{-1}$] by the α-195^{Gln} MoFe Protein by CO

conditions	H_2 evolution	ATP:2e$^-$
argon (101 kPa)	2810	5.5
argon (101 kPa) and 20 mM azide	615	29.3
argon (100 kPa) and CO (1 kPa)	2985	4.9
argon (100 kPa), 20 mM azide, and CO (1 kPa)	3006	4.8

Addition of "azide" to complete reaction mixtures containing the α-195^{Gln} MoFe protein results in a marked effect on the ATP:2e$^-$ ratio, which increases from ca. 5 for H_2 evolution under argon in the absence of "azide" to ca. 18 at 3 mM and ca. 28 at 10 mM "azide" (Figure 1). This increase is largely due to pronounced inhibition of H_2 evolution that is not compensated for by other product formation (Table 1). For example, H_2 evolution is 74% inhibited at 5 mM azide (see Table 2). The increase in the ATP:2e$^-$ ratio is compounded by a moderate stimulation of ATP hydrolysis. In three separate experiments, the mean rate of ATP hydrolysis increased ($p < 0.001$) from 15.4 ± 1.4 in the absence of azide to 19.9 ± 1.4 μmol min^{-1} (mg of MoFe protein)$^{-1}$ at 3 mM azide. "Azide" inhibition of electron flow to product with the α-195^{Gln} MoFe protein is fully reversible by addition of 1 kPa CO (Table 2). CO also completely inhibits N_2H_4 and NH_3 formation from "azide" by the α-195^{Gln} MoFe protein (data not shown) as it does for the wild type.

Table 3: Effect of pH on the Degree of Inhibition by "Azide" of H_2 Evolution by the α-195^{Gln} MoFe Protein

	1 mM azide			5 mM azide		
	$[HN_3]$ (μM)	$[N_3^-]$ (μM)	% inhibition	$[HN_3]$ (μM)	$[N_3^-]$ (μM)	% inhibition
pH 7.15	2.8	997	48	14	4986	70
pH 6.78	6.6	994	55	33	4967	74
pH 6.50	12.4	988	56	62	4938	75
pH 6.20	24.5	976	61	122	4877	77

pH Effects on "Azide" Reduction. Inhibition of H_2 evolution was monitored in assays, which contained total "azide" concentrations of either 1 or 5 mM, when the pH was varied over the range of 7.15−6.20. Because HN_3 is a weak acid with a pK_a of 4.6 at 30 °C, the HN_3 concentrations vary from 2.8 to 24.5 μM for 1 mM total "azide" and from 14 to 122 μM for 5 mM "azide" over this pH range. In contrast, the N_3^- concentrations vary very little over this pH range, from 0.99 to 0.98 mM and from 4.99 to 4.88 mM for the two "azide" concentrations, respectively. Data for H_2 evolution are presented in Table 3. The degree of inhibition varies little with pH at either "azide" concentration, which suggests that the inhibition is due to N_3^-. From these observations, the rate of N_2H_4 formation as a function of HN_3 concentration yields a linear Lineweaver−Burk plot giving a value for the K_m of 10 μM, very similar to the value reported for the wild-type MoFe protein of 12.2 μM (*34*). Measurement of the inhibition of H_2 evolution as a function of "azide" concentration leads to "azide" concentrations of 0.7−0.9 mM for half-maximal inhibition, compared to K_m values of 1.1 mM "azide" for NH_3 formation and 10−12 μM HN_3 (equivalent to ca. 3.6 mM "azide" at pH 7.30) for N_2H_4 production.

Is the Inhibition of H_2 Evolution by "Azide" Reversible? The extent of H_2 evolution by the α-195^{Gln} MoFe protein was measured in either 0.2 mL reaction mixtures, which contained twice the normal creatine phosphate concentration with either 0.2 or 2 mM "azide", or 2 mL reaction mixtures with the same composition (Figure 2). Up to 6 min, the two different-sized assay volumes produced the same rate of H_2 evolution, although the rate with 2 mM "azide" was only 40% of that with 0.2 mM "azide". At 4 min, some of the 0.2 mL assays containing 2 mM "azide" were diluted 10-fold with an assay mixture with the same composition but lacking "azide", and further H_2 evolution was monitored. The 10-fold decrease in "azide" concentration resulted in an immediate switch to a rate of H_2 evolution characteristic of the 0.2 mM "azide" assays, indicating that "azide" inhibition is readily reversible.

Is "Azide" Inhibition of Electron Flux Mediated by N_2 Produced from N_3^- Reduction? Because D_2 (and H_2) relieves N_2 inhibition of electron flow to substrate with the α-195^{Gln} MoFe protein (see below), H_2 evolution was measured in assays containing "azide" under an atmosphere of D_2. The data in Table 4 show that D_2 did not relieve "azide" inhibition of H_2 evolution. It is, therefore, unlikely that "azide" inhibition of electron flow is due to N_2 produced from N_3^- reduction. No HD formation was observed with either the α-195^{Gln} or wild-type MoFe proteins in the presence of both "azide" and D_2 despite the fact that N_2 was being produced, albeit at very low concentrations for the α-195^{Gln} MoFe protein.

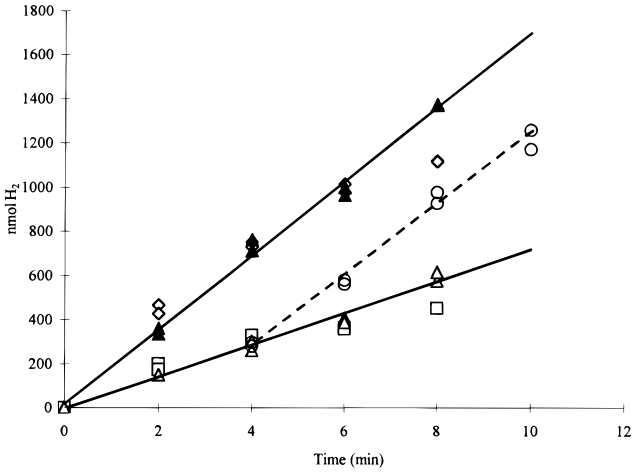

FIGURE 2: Reversibility of "azide" inhibition of H_2 evolution from the α-195^{Gln} MoFe protein. H_2 evolution catalyzed by a constant amount (0.071 mg) of α-195^{Gln} MoFe protein was measured at 0.2 mM NaN_3 (◇ and ▲) or 2 mM NaN_3 (□ and △) in either a 0.2 mL assay (◇ and □) or a 2.0 mL assay (▲ and △) containing twice the normal concentration of creatine phosphate. After 4 min, 1.8 mL of the assay mixture with the same composition but lacking "azide" was injected into six 0.2 mL assays (○) containing 2.0 mM NaN_3, and H_2 evolution followed for a further 6 min. The rate of H_2 evolution shifted from 65 nmol min^{-1} at 2 mM NaN_3 to 158 nmol min^{-1}. The control rate at 0.2 mM NaN_3 was 161 nmol min^{-1}.

Table 4: Effect of D_2 on the "Azide" Inhibition of Electron Flux with the α-195^{Gln} MoFe Protein [rate of product formation in nmol min^{-1} (mg of MoFe protein)$^{-1}$]

conditions	H_2	N_2	N_2H_4	NH_3
argon	2816			
argon and 10 mM KN_3	679	53	8.2	78
D_2	2836			
D_2 and 10 mM KN_3	641	44	8.9	74

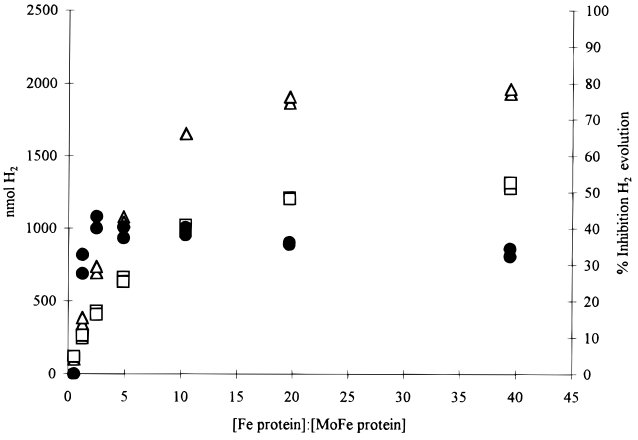

FIGURE 3: H_2 evolution by the α-195^{Gln} MoFe protein as a function of the Fe protein:MoFe protein ratio under an atmosphere of argon (△) or dinitrogen (□) with the percentage inhibition by N_2 (●). The concentration of the α-195^{Gln} MoFe protein was kept constant at 0.072 mg per 1 mL assay, and the protein was titrated with increasing amounts of the Fe protein (from 0.01 to 0.82 mg). The assay time was 10 min.

Component Protein Effect on N_2 Inhibition of H_2 Evolution. Varying the ratio of Fe protein to α195^{Gln} MoFe protein between 0.5:1 and 40:1 and under either 101 kPa Ar or 101 kPa N_2 showed that the amount of evolved H_2 increased in a similar pattern for both (Figure 3) with a half-maximal rate of H_2 evolution occurring at a ratio of 8 under Ar and 6 under N_2. The percentage inhibition of H_2 evolution by

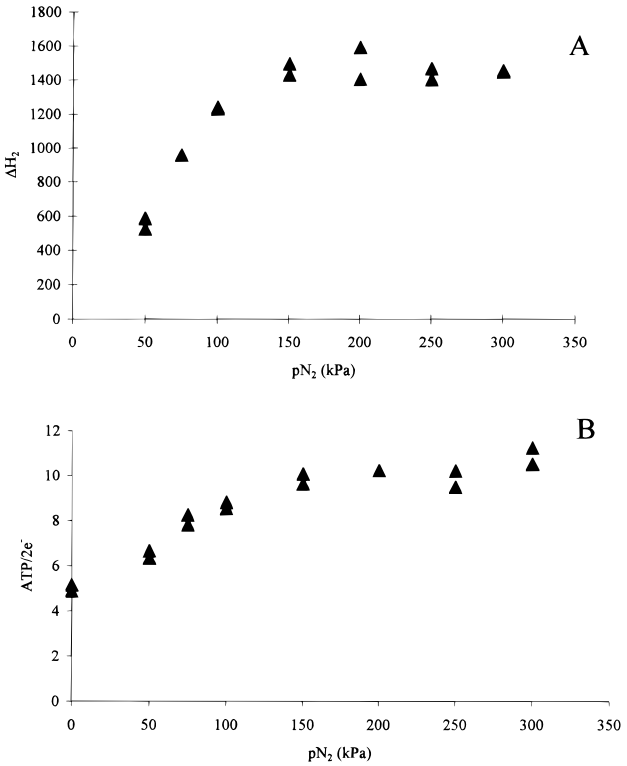

FIGURE 4: (A) Inhibition of H_2 evolution (ΔH_2, nanomoles) by the α-195^{Gln} MoFe protein as a function of pN_2. The standard hyperbaric assay system contains 0.071 mg of α-195^{Gln} MoFe protein at a 20:1 Fe protein:MoFe protein molar ratio. (B) ATP:$2e^-$ ratio for assays of the α-195^{Gln} MoFe protein with varying pN_2 values.

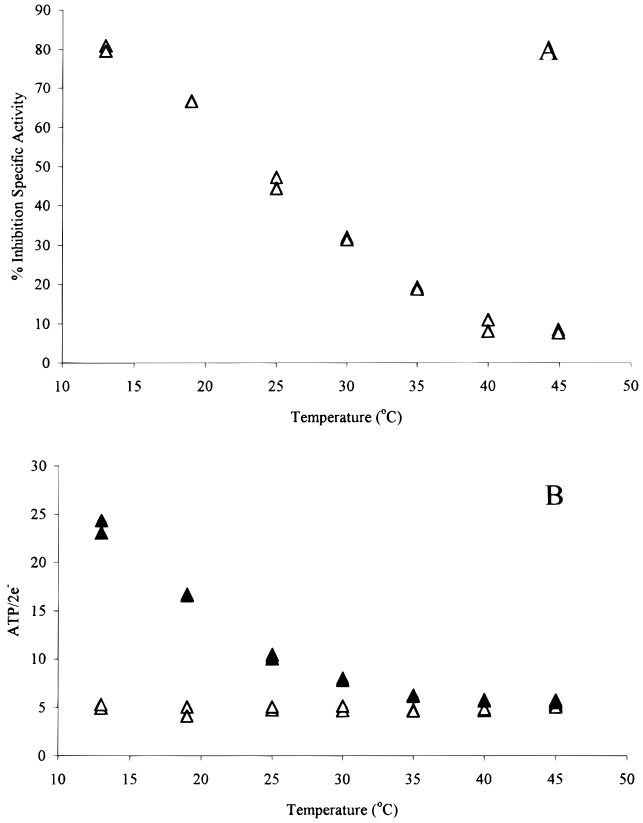

FIGURE 5: (A) Percentage inhibition of H_2 evolution by the α-195^{Gln} MoFe protein under N_2 vs Ar as a function of temperature. Steady-state assays were performed at a 20:1 Fe protein:MoFe protein molar ratio. (B) ATP:$2e^-$ ratio for assays of the α-195^{Gln} MoFe protein under N_2 (▲) or Ar (△) as a function of temperature.

N_2 increased from zero at an Fe protein:MoFe protein ratio of 0.5:1 to ca. 30% at 2.5:1 and remained almost constant thereafter up to a ratio of 40:1. With wild-type nitrogenase, the major inhibitory effect (ca. 85% of the total achievable inhibition) of N_2 on H_2 evolution is also maximal at an Fe protein:MoFe protein ratio of ca. 2.5:1, although a minor contribution to inhibition continues to a ratio of greater than 10:1.

Effect of pN_2 on Inhibition of H_2 Evolution and on the ATP:$2e^-$ Ratio. In earlier work (*1*), the affinity of N_2 for the α-195^{Gln} MoFe protein was estimated by measuring the $K_i(N_2)$ for the inhibition by N_2 of the reduction of C_2H_2 to C_2H_4 and deriving a value of 40 kPa. We have estimated this parameter directly by measuring the loss of H_2 evolution activity as a function of pN_2 up to 303 kPa. Figure 4A shows that the decrease in the amount of evolved H_2 appears to follow Michaelis−Menten kinetics, from which a K_m of 65 kPa N_2 can be calculated. The ATP:$2e^-$ ratio reaches a plateau of about 10 at about 150 kPa N_2 (Figure 4B), a value that is approximately double that under Ar.

Reduction of N_2 to NH_3. Because only very low rates, at best, of NH_3 production were expected, a new method (*55*) was developed to measure very small amounts of NH_3 in the presence of the large amounts of creatine produced in assays of this highly uncoupled altered MoFe protein. Hyperbaric N_2 pressures were also used to increase the yield. The results showed that, at 202 kPa N_2, the α-195^{Gln} nitrogenase does reduce N_2 to NH_3 at a rate of 23 nmol min^{-1} (mg of MoFe protein)$^{-1}$, a value that is 1−2% of that for the wild type. Its apparent K_m for NH_3 formation is very high (380 kPa). This ammonia production was completely inhibited by addition of either 2 kPa CO or 101 kPa H_2. No N_2H_4-producing intermediate (*25*) was detected when substrate amounts of the α-195^{Gln} MoFe protein were quenched by added acid during turnover. In a parallel experiment, 20 μM wild-type MoFe protein produced a maximum of 2.5 nmol of N_2H_4. If the rate of formation of this intermediate by the α-195^{Gln} MoFe protein is assumed to reflect the substantially decreased rate of NH_3 formation, detection of 1−2% of the wild-type amount of N_2H_4 would not have been feasible under the conditions used.

Temperature Effects on N_2 Inhibition of H_2 Evolution and the ATP:$2e^-$ Ratio. Under normal assay conditions at 30 °C, even 303 kPa N_2 produces only about a 50% inhibition of H_2 evolution. However, the degree of inhibition is very strongly affected by temperature. The data in Figure 5A show that the degree of N_2 inhibition of H_2 evolution increases to 80% at 13 °C. They also show that increasing the temperature to 45 °C almost eliminates the inhibitory effect of N_2 (ca. 8% inhibition). The ATP:$2e^-$ ratio plots over this temperature range (13−45 °C) (Figure 5B) show that, under Ar, the value remains constant, whereas under N_2, it falls from nearly 25 to a value not significantly different from that under Ar. Although the differences are small and disappear at 45 °C, the rate of ATP hydrolysis is significantly increased under N_2 ($p < 0.001$). At 35 °C, the rate of ATP hydrolysis increases from 22.1 ± 0.72 to 24.4 ± 0.55 μmol min^{-1} (mg of MoFe protein)$^{-1}$ under 101 kPa argon versus 101 kPa N_2, respectively.

Table 5: Prevention by H_2 of N_2 Inhibition of Acetylene Reduction with Wild-Type and α-195^{Gln} MoFe Proteins

MoFe protein	assay atmosphere	acetylene reduction rate [nmol min^{-1} (mg of MoFe protein)$^{-1}$]	relative activity[a] (%)
wild-type	0.5 kPa C_2H_2 and 102 kPa Ar	705	100
	0.5 kPa C_2H_2, 62 kPa N_2, and 40 kPa Ar	288	41
	0.5 kPa C_2H_2, 62 kPa N_2, and 40 kPa H_2	553	78
α-195^{Gln}	0.5 kPa C_2H_2 and 102 kPa Ar	740	100
	0.5 kPa C_2H_2, 62 kPa N_2, and 40 kPa Ar	507	68
	0.5 kPa C_2H_2, 62 kPa N_2, and 40 kPa H_2	743	100

[a] Specific activity under C_2H_2/Ar set at 100.

Table 6: Formation of H_2, HD, and NH_3 by Wild-Type and α-195^{Gln} MoFe Proteins

		specific activity [nmol of electron pairs min^{-1} (mg of MoFe protein)$^{-1}$][a]				percentage of electron allocation[b]			
MoFe protein	atmosphere	H_2	HD	NH_3	total	H_2	HD	NH_3	ATP:2e$^-$
wild-type	102 kPa Ar	2291	0	0	2291	100	0	0	4.3
	51 kPa D_2 and 51 kPa N_2	1074	615	700	2389	45	26	29	4.7
α-195^{Gln}	102 kPa Ar	2574	0	0	2574	100	0	0	5.0
	51 kPa D_2 and 51 kPa N_2	1748	735	24	2507	70	29	1	4.3

[a] One, two, or three electrons allocated for HD, H_2, and NH_3 formation, respectively. [b] Percentage electron allocation calculated as electron flux to each product divided by the total electron flux.

Restoration of Electron Flux for the α-195^{Gln} MoFe Protein. H_2 is a specific inhibitor of the wild-type reduction of N_2. If H_2 relieved the N_2 inhibition of electron flow to substrate with the α-195^{Gln} MoFe protein, it would indicate that the interaction of N_2 with the altered MoFe protein was equivalent to that for the wild type. Because only very small quantities of NH_3 are produced, we had to resort to indirect methods to investigate this possibility. We chose to study the effect of H_2 on the inhibition by N_2 of acetylene reduction. The results in Table 5 show that, when a relatively low concentration of C_2H_2 is used, 62 kPa N_2 inhibits C_2H_4 production with both wild-type and α-195^{Gln} MoFe proteins, and that introduction of 40 kPa H_2 reverses this trend partially for the wild type and completely for the α-195^{Gln} MoFe protein.

HD Formation. In a similar experiment, where 51 kPa D_2 was added to 51 kPa N_2 and products were quantified by mass spectrometry, total electron flow through the α-195^{Gln} nitrogenase was fully restored (Table 6). The same experiment also showed that the α-195^{Gln} nitrogenase carried out N_2-catalyzed HD formation at a rate very similar to that for the wild-type enzyme under the same conditions. No HD was detectable for either MoFe protein under an Ar/D_2 atmosphere (data not shown). The percentage of total electron flow allocated to HD formation was also similar for the two enzymes. However, for the α-195^{Gln} nitrogenase, these electrons corresponded to those recovered by relieving the inhibition of electron flow caused by the presence of 51 kPa N_2 rather than to a decrease in electron flow to NH_3, which is typical for wild-type N_2-catalyzed HD formation (*37*, *44*). For the α-195^{Gln} nitrogenase, the level of electron flow to NH_3 is so low that HD formation cannot be explained in the same way.

Cyanide Reduction. Data for H_2 evolution and CH_4 production from cyanide were summed, and the total amount of electron flow to all products was calculated as described previously (*26*; see Experimental Procedures). Wild-type MoFe protein from *A. vinelandii* showed the expected decline in the rate of substrate reduction (Figure 6) with increasing cyanide concentration, whereas the rate with the α-195^{Gln} nitrogenase was clearly unaffected by increasing cyanide levels. The specific activities of the α-195^{Gln} and wild-type MoFe proteins for CH_4 production, 263 and 121 nmol min^{-1} (mg of protein)$^{-1}$, respectively, at 5 mM cyanide, were different as a consequence of the approximately 60% inhibition of electron flux through the wild-type protein. Data (not shown) for the ATP:2e$^-$ ratio are constant (6.0 ± 0.5) for cyanide concentrations up to 10 mM for the α-195^{Gln} MoFe protein, but increased from 5.0 ± 0.5 at no added cyanide to 25 ± 1.0 at 25 mM cyanide for the wild-type MoFe protein as reported previously (*26*).

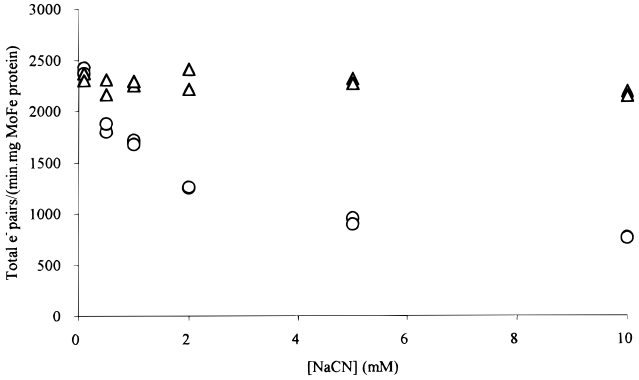

FIGURE 6: Total amount of electron flow to substrate through wild-type (○) and α-195^{Gln} (△) MoFe proteins as a function of NaCN concentration. The total amount of electron flow was calculated from the rates of H_2 and CH_4 evolution [(2 × H_2) + (7.88 × CH_4); *26*].

DISCUSSION

Because the α-195^{Gln} nitrogenase reduces protons and C_2H_2 at rates which are not markedly different from those for the wild-type enzyme (*1*), it was expected that "azide" would also be reduced at an appreciable rate by this altered enzyme, but this was found not to be the case. The rates observed were only about 5−7% of those for the wild type. Unlike the rates for most wild-type nitrogenase-catalyzed reactions, these low rates of "azide" reduction are not compensated for by a high H_2 evolution rate, so "azide" acts

as an effective inhibitor of electron flow to substrate and increases the ATP:$2e^-$ ratio from ca. 5 (for H_2 evolution under argon) to ca. 28 at 10 mM "azide". This reactivity pattern resembles the effect of N_2 on this altered protein, where there is a similar level of inhibition of H_2 evolution without a decrease in the level of ATP hydrolysis (*1*). Although the level of N_2 evolution from N_3^- reduction was not directly measured, the flux attributable to N_3^- reduction is only 13% of the residual H_2 evolution (Table 1), so any N_2 produced cannot possibly account for the massive decrease observed in the total amount of electron flow with "azide".

It has been reported (*34*) that the total amount of electron flow through wild-type nitrogenase was unaffected by "azide" at concentrations up to 20 mM, provided the pH was $\leq$7.3, but that electron flow was weakly (ca. 25%) inhibited at pH 7.6 by "azide" at concentrations of >5 mM. The extent of inhibition (74%) noted here with the α-195^{Gln} nitrogenase is much greater. Further, the "azide" inhibition of electron flow noted with the wild-type MoFe protein from *A. vinelandii* appeared nonspecific because, unlike the CN^- inhibition of electron flow to substrate (*26*), it was not reversible by addition of CO (*34*). With the α-195^{Gln} nitrogenase, CO completely inhibits N_2H_4 and NH_3 formation from "azide" and reverses fully "azide" inhibition of electron flow. "Azide" inhibition of electron flow was also easily reversed by simple dilution. "Azide" inhibition of H_2 evolution is, therefore, not due to a nonspecific inactivation of the altered MoFe protein. Another effect of "azide" with the α-195^{Gln} nitrogenase is a moderate stimulation of ATP hydrolysis, which suggests an additional interaction of "azide" at a site distinct from that for reduction of this substrate.

The nature of the inhibitory species in "azide" solutions, N_3^- or HN_3, was addressed by monitoring how pH affects the inhibition of H_2 evolution by "azide". The degree of inhibition varied little with pH at the "azide" concentrations used. This result suggested that the inhibition of H_2 evolution is due to N_3^-, the concentration of which varies very little with pH, and not HN_3, the concentration of which varies 9-fold across this pH range. Because N_2 is a product of the limited N_3^- reduction catalyzed by the α-195^{Gln} MoFe protein and because N_2 reversibly inhibits H_2 evolution without being rapidly reduced by this altered MoFe protein (*1*), a logical question is whether the "azide" inhibition is mediated by N_2. This possibility appeared unlikely because, at 30 °C, the inhibition of H_2 evolution by even 2 mM "azide" is much more severe than that produced by 101 kPa N_2 and very little N_2 is produced by "azide" reduction. However, one unexplained aspect of "azide" reduction is that the N_2 produced by the wild-type MoFe protein appears not to be in equilibrium with N_2 in the environment (*34*) but the amount of N_2 reduced further to NH_3 is much greater than can be accounted for by the very low pN_2 generated by "azide" reduction. This seeming paradox has been rationalized by suggesting that the site where N_2 is released from N_3^- reduction is very close to that where N_2 is reduced (*34*) so that localized high N_2 concentrations result in the high degree of inhibition observed with "azide". This question was investigated through the effects of adding H_2 (or D_2) to N_2-inhibited assays. Because, with the wild-type enzyme, H_2 inhibits the binding and reduction of N_2 only, it was reasonable to ask whether D_2 would similarly relieve "azide" inhibition of electron flux. Although H_2 (or D_2) was found to relieve both the inhibition by N_2 of C_2H_2 reduction and the N_2 inhibition of electron flux with the α-195^{Gln} MoFe protein, D_2 did not relieve "azide" inhibition of H_2 evolution. It is, therefore, very unlikely that "azide" inhibition of electron flux is due to N_2 produced from N_3^- reduction.

Another major surprise was the formation of "excess" ammonia, that is, ammonia over and above that expected from the direct reduction of "azide". The formation of "excess" ammonia from the nitrogenase-catalyzed reduction of "azide" has been rationalized previously (*33*, *34*) as being due to further reduction of N_2 formed from N_3^-. Its production by the α-195^{Gln} MoFe protein was totally unexpected because this altered protein has been reported (*1*) to be unable to reduce N_2 to ammonia (but see below). H_2 at 101 kPa has been reported to partially eliminate "excess" ammonia production from "azide" (*34*) and also to completely inhibit N_2 reduction to NH_3 with wild-type nitrogenase. However, "excess" ammonia production from "azide" by the α-195^{Gln} MoFe protein is totally unaffected by the presence of 101 kPa H_2. The lack of an effect of H_2 on both "excess" ammonia production and on "azide" inhibition of electron flow to substrate suggests that these two reactions are separate and distinct from any reaction involving N_2.

With wild-type MoFe proteins from both *Klebsiella pneumoniae* and *A. vinelandii*, N_2 is a weak competitive inhibitor of HN_3 reduction to N_2H_4 (*33*, *34*) with a K_i of 0.42 atm for the *K. pneumoniae* MoFe protein. With the α-195^{Gln} MoFe protein, N_2 is also a weak inhibitor (data not shown), but the small quantities of N_2H_4 produced make determination of the type of inhibition difficult. Further, any pattern of inhibition may not be readily interpreted because the presence of N_2 accentuates the inhibition of electron flow already exerted by "azide" and increases the ATP:$2e^-$ ratio. The increase in the ATP:$2e^-$ ratio caused by N_2, even at "azide" concentrations already causing 77% inhibition of H_2 evolution, reinforces the suggestion that the inhibitory effects of "azide" and N_2 on electron flux are unrelated.

The multitude of possibilities described above for N_2 involvement in the catalyzed reduction of "azide" led us to reconsider the interactions of N_2 with the α-195^{Gln} MoFe protein. Because we wanted to gain insight into the mechanisms of both N_2 inhibition of H_2 evolution and HD formation under a N_2/D_2 atmosphere, it was critical to reinvestigate whether this α-195^{Gln} MoFe protein could achieve sufficiently reduced states of complexed N_2 to produce NH_3. Using a modified colorimetric assay (*55*) to measure very small amounts of NH_3 in the presence of the large amounts of creatine and using hyperbaric N_2 pressures to increase yields, it was found that the α-195^{Gln} nitrogenase indeed reduces N_2 to NH_3 but at a rate that is only 1−2% of that for the wild type. Most importantly, this ammonia production was completely inhibited by either CO or H_2. The possibility that the NH_3 produced from N_2 results from adventitiously copurified traces of an alternative nitrogenase is remote for two reasons. First, the cells were grown at a high molybdate concentration (10 μM Na_2MoO_4), which is sufficient to repress the synthesis of alternative nitrogenases in Δ*nifHDK* strains of *A. vinelandii*. Second, all known alternative nitrogenases catalyze the reduction of acetylene

to a mixture of ethylene and ethane, whereas the α-195^{Gln} nitrogenase produces no detectable ethane.

Although the rate of N_2 *reduction* catalyzed by the α-195^{Gln} MoFe protein is much lower than that of wild type, the *binding* of N_2 appears to be comparable to that for the wild-type MoFe protein. Notably, N_2 inhibition of H_2 evolution becomes maximal at the relatively low Fe protein:MoFe protein ratio of ca. 2.5 for both nitrogenases. Given that a greater Fe protein:MoFe protein ratio offers a greater opportunity for more highly reduced states of the enzyme to be achieved, these data suggest that the site binding N_2 and inhibiting H_2 evolution is sufficiently reduced to do so at a 2.5:1 Fe protein:MoFe protein molar ratio for both the wild type and this altered nitrogenase; i.e., N_2 *binding* does not require a very highly reduced state of the FeMo cofactor within the MoFe protein.

Under a mixed N_2/D_2 atmosphere, the α-195^{Gln} nitrogenase catalyzes the production of HD at a rate as rapid as that for the wild type (Table 6). Rapid HD formation and the very low rate of N_2 reduction and the failure to detect the N_2H_4-producing species on acid quenching, which shows how low the concentration of protonated nitrogen species must be with this protein under these conditions, make it improbable that any diazene level species is a part of the mechanism of HD formation. It would appear that bound N_2 is sufficient. With wild-type nitrogenase, electrons used for HD formation are diverted from those intended for NH_3 formation, the rate of which therefore decreases (*37*, *44*). In contrast, with the α-195^{Gln} nitrogenase, the electrons producing HD apparently are those electrons whose appearance as H_2 has been suppressed by N_2. When N_2 inhibition of H_2 evolution with both wild-type and α-195^{Gln} nitrogenase is considered, it becomes clear that, in both cases, the presence of N_2 serves to divert electron flow from H_2 evolution. In the wild type, these diverted electrons go to produce NH_3, whereas with the α-195^{Gln} MoFe protein, they form no product. In neither case, however, does N_2 divert (or inhibit) all electron flow away from H_2 evolution, and in both cases, the presence of H_2 (D_2) restores total electron flow to H_2 evolution. Labilization of hydrides bound to a metal center by the binding of a N_2 molecule to a separate site on the FeMo cofactor (*56*) could account for these observations.

A corollary of these observations may be that H_2 evolution occurs from more than one site: from the site(s) of N_2 binding and from a site elsewhere on the cluster. If so, the proportions of H_2 coming from these two sites, one N_2-binding and the other not, must vary with temperature because the degree of inhibition of H_2 evolution by N_2 varies with temperature from almost zero at 45 °C to ca. 80% at 13 °C. Over this same temperature range, the ATP:2e$^-$ ratio also increases from a value close to normal to nearly 25. These data suggest strongly that conformational changes resulting from the change in temperature profoundly modify the ability of bound N_2 to influence the rates of either electron transfer to H^+ or ATP hydrolysis or both. Moreover, although the differences are small and disappear at 45 °C, the rate of ATP hydrolysis is significantly increased under 101 kPa N_2 versus 101 kPa argon. This temperature effect may be of use in spectroscopic probing of the nitrogenase FeMo cofactor because N_2 may be complexed more firmly at lower temperatures, thereby facilitating use of techniques such as either FTIR or ^{15}N NMR.

The reason for the increased rate of ATP hydrolysis during either N_2 or N_3^- inhibition of H_2 evolution is difficult to identify. Whereas an increased level of ATP hydrolysis with N_3^- might be attributable to N_3^- complexation to iron−sulfur centers other than the FeMo cofactor, this explanation seems improbable for the effect of N_2 because all data acquired, i.e., the relief of the inhibitory effects of N_2 by CO or H_2 and HD formation, are consistent with the inhibitory effects of N_2 being mediated through its normal mode of binding. Why then does the rate of ATP hydrolysis increase with the α-195^{Gln} MoFe protein, whereas electron transport to H^+ is inhibited by N_2 binding? A simplistic answer is that, when the MoFe protein becomes "saturated" with electrons due to an inhibition of the rate of substrate reduction (for whatever reason), transfer of subsequent electrons from the Fe protein to the MoFe protein becomes more expensive energetically. Such an increase in the level of MgATP use would also be reflected by an increased ATP:2e$^-$ ratio.

An alternative explanation could have its basis in the suggestion that, once an appropriate complex has been formed among the Fe protein, the MoFe protein, and MgATP, ATP hydrolysis is inevitable even if electron transfer does not occur (*13*). In the current situation then, the so-called reductant-independent ATP hydrolysis, ATP hydrolysis unaccompanied by productive electron transfer, could be invoked as an explanation. This phenomenon has been observed when the dye-oxidized Fe protein becomes complexed to the reduced MoFe protein, but it does not occur with either protein alone (*57*). This complex is capable of being recharged with two molecules of MgATP without dissociation of the proteins (*13*). It is not clear, however, if this process can occur simultaneously with ATP hydrolysis associated with electron transfer. If it can, then stimulation of reductant-independent ATP hydrolysis by the binding of either N_2 or N_3^- to the α-195^{Gln} MoFe protein would explain our observations. However, this explanation would require that both of these substrates specifically stimulate this process only when electron transfer is inhibited by their binding. Unfortunately, these are conditions whose occurrence we cannot evaluate.

"Futile cycling" of electrons between the Fe protein and the MoFe protein accompanied by ATP hydrolysis (*58*, *59*) has been offered as another explanation of continued ATP hydrolysis under conditions, such as an unfavorable component protein ratio, pH, or temperature, where electron transfer is limited. We do not favor such cycling as an explanation for our observations because it presumably requires a longer association time between the Fe protein and MoFe protein to allow the electron to be transferred to the MoFe protein and then to return to the Fe protein. Therefore, it should result in decreased absolute rates of ATP hydrolysis, even though the ATP:2e$^-$ ratio would increase. Moreover, if futile cycling were to be operative in the Fe protein−α-195^{Gln} MoFe protein complex, the binding of either N_2 or N_3^- at the modified FeMo cofactor would have to have a long-range influence on the association−dissociation kinetics of the two proteins. There appears to be no evidence to support this hypothesis.

An additional consideration comes from the published observation that electron transfer to a metal center embedded in a protein appears to be more facile if a proton can be transferred with that electron (see, for example, ref *60*). Thus,

if proton translocation to the FeMo cofactor occurs via the α-histidine-195 residue during both N_2 and azide reduction, the α-glutamine-195 substitution may be disruptive. This interpretation has the further implication that, because other substrates are reduced normally, additional channels exist to deliver protons to different sites on the FeMo cofactor for reduction of other substrates. This clear differentiation among substrates, for example, C_2H_2 reduction and N_2 reduction, has been observed before with the β-98^{His} MoFe protein. This altered MoFe protein was suggested to suffer from a defect in intra-MoFe protein electron transfer specifically for C_2H_2 reduction (*61*). If so, then it is possible not only that individual (or subgroups) substrates and inhibitors have their own interaction site but also that they also have customized electron-transfer and proton-transfer pathways.

The interactions of CO and CN^-, both of which are potent, but distinct, inhibitors of wild-type nitrogenase, with the α-195^{Gln} MoFe protein are also instructive. CO inhibits the reduction of all substrates except H^+ with wild-type nitrogenase. It does not inhibit electron flow through the MoFe protein but acts to divert all electrons to H_2 evolution; thus, ATP hydrolysis and product formation remain tightly coupled. Substitution of glutamine for α-195-histidine has only a minor effect on the CO interaction (*1*). In contrast, CN^- is a potent inhibitor of total electron flow to substrate (by ca. 60% at 5 mM cyanide) through the wild-type MoFe protein during turnover, resulting in an uncoupling of ATP hydrolysis from electron transfer to substrate. With the wild type, CO has been reported to relieve totally the inhibition by CN^- of electron flow to substrate, thereby implying a common binding site (*26*). We have found that CN^- is completely ineffective with the α-195^{Gln} nitrogenase, even though HCN is actively reduced, with the result that ATP hydrolysis remains tightly coupled to electron transfer even at 10 mM cyanide. The binding site for CN^- has obviously been affected dramatically by the His-to-Gln substitution at the α-195 position. These results further suggest that these two inhibitors are unlikely to share a common binding site. The efficient reduction of HCN was particularly surprising in light of the low activities for N_3^- and HN_3 reduction catalyzed by the α-195^{Gln} MoFe protein because azide has been reported to be a competitive inhibitor of HCN reduction (*32*, *62*).

In conclusion, in attempting to analyze and understand the changes that the substitution of the α-195-histidine by glutamine has caused and what implications these have for the mechanism of nitrogenase action, we can start from the knowledge that the imidazole ϵ-nitrogen of histidine and the amide group nitrogen of glutamine can be disposed similarly with respect to the FeMo cofactor surface because the chain length from the backbone α-carbon to these nitrogens is the same for both residues. The putative hydrogen bonding to a central S atom is, therefore, likely to be maintained in both MoFe proteins. It follows then that the glutamine substitution at position α-195 produces only very local effects on the electronic structure of the FeMo cofactor. This suggestion is supported by the observation of only minor shifts in *g* values in the corresponding EPR signals (*1*, *63*). Thus, it might be predicted that the catalyzed reduction of only those substrates which interact with the central girdle of six iron and three S atoms would be affected by this substitution. Those substrates and inhibitors affected by the substitution of α-195-histidine with glutamine are N_2, N_3^-, HN_3, and CN^-. It is tempting to speculate that these molecules bind in this area of the FeMo cofactor and that H^+, C_2H_2, HCN, and CO do not.

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