**SUPPLEMENTARY INFORMATION**

**Mutant strain construction**. Oligonucleotides were purchased from DNAgency. Site-directed mutagenesis, gene replacement and the isolation of mutant strains carrying substitutions at either the $\alpha 277^{Arg}$ or $\alpha 192^{Ser}$ position were performed as described or cited previously.[1,2] Strains containing substitutions at the $\alpha 356^{Gly}$ position were constructed using the Altered Sites II *in vitro* mutagenesis system (Promega). A 2.9-kb Sal1 fragment, pDB14, containing the *nifHDK* fragment was cloned into the Sal1 site of the pALTER-1 vector. Double stranded DNA was isolated[3] and single-stranded DNA was prepared with R408 as helper phage.[4] A 27-mer oligonucleotide containing a change in the central codon was annealed to the single stranded template DNA in order to introduce the desired substitution; the Altered Sites II procedure was then followed. A wild type (Nif$^+$) strain of *A. vinelandii* was transformed by congression with pDB303, a plasmid carrying *rif* resistance, and selection was for the loss of diazotrophy.

**Cell growth and nitrogenase derepression**. Wild-type and mutant strains were grown on urea-supplemented Burk's media as described previously,[5] including larger batches grown in a MF-128S 28-L fermentor (New Brunswick Scientific Co., NJ). At a turbidity of *ca.* 200 Klett units, using a Klett-Summerson colorimeter equipped with a no.54 filter (Klett Manufacturing Co., NY), cells were concentrated to 2 L inside the fermentor by filtration with a Millipore (Pellicon) 0.45μm cassette connected to a Watson–Marlow 601S/R peristaltic pump. Cells were then washed with 2 x 2.5-L additions of phosphate buffer to remove any residual urea. The cell culture was restored to 24 L with Burk's media and allowed to derepress for 3.5 hrs. After a second filtration-concentration step, cells were harvested by centrifugation in a Sorvall RC-5B refrigerated centrifuge at 6,000 x g and 4°C for 10 minutes. The resulting cell paste was stored at –80°C until needed.

**Crude-extract preparation and nitrogenase purification**.  Anaerobically thawed whole cells were resuspended at 4°C in two volumes of degassed 100 mM Tris-HCl buffer (pH 8.0) containing 2 mM sodium dithionite. The resulting cell suspension was disrupted in a 200-mL Ar-flushed Rossette cooling cell (Heat systems-Ultrasonics, NY) submerged in an ice/water bath by six one-minute sonication cycles, separated by one-minute cooling intervals, using a Heat System Sonicator model XL2015. The disrupted extract was transferred to an anaerobic flask containing 10 mg $L^{-1}$ DNase and RNase, a 30% anaerobic streptomycin sulfate solution was then added to give a final concentration of 1% w/v and the solution stirred for 30 minutes at room temperature. Cellular debris was removed by centrifugation at 98,000 x g for 90 minutes. The supernatant was frozen and stored in liquid $N_2$ until used.

Nitrogenase component proteins were purified from crude extracts as described previously,[6] except that Q-Sepharose rather than DEAE-cellulose anion-exchange chromatography was used and Sephacryl S-300 gel filtration was performed prior to and in addition to Sephacryl S-200 gel filtration. Because none of the variant MoFe proteins could be purified by crystallization, a final purification step employed phenyl-Sepharose hydrophobic-interaction chromatography.[7] All purified nitrogenase proteins were dialyzed into 25 mM HEPES buffer (pH 7.4) before use.

**Protein estimation, gel electrophoresis and metal analysis**.  Protein concentrations were determined[8] using bovine serum albumin as a standard. The purity of the component proteins was monitored by SDS-PAGE,[9] stained with Coomassie Blue R-250. The Mo and Fe contents of the purified MoFe proteins were determined using a Perkin-Elmer 400 Inductively Coupled Plasma Emission spectrometer and are listed in Table S3.

**Nitrogenase activity assays and product analysis**.  Fe-protein and MoFe-protein specific activities were determined at each stage of purification in the presence of an optimal amount of

the purified complementary protein as described previously.[7] All assays were stopped by addition of 0.3 mL of 0.5 M $Na_2EDTA$ (pH 7.5). Acetylene was prepared from the action of water on calcium carbide (Fisher Scientific, PA) and added to the assay vial during the pre-incubation period with a gas-tight syringe. CO was added similarly. For experiments requiring very low CO concentrations, a serial dilution with Ar was performed before addition. All data shown are representative of at least three individual experiments and all data points were measured in duplicate. To conduct assays at pH values other than the standard pH 7.4, a three-component buffer system was used.[10] Gaseous products were analyzed and quantified by gas chromatography of 200-$\mu$L aliquots from the reaction vials as previously described.[7] MgATP hydrolysis in assays containing a creatine phosphokinase ATP-regenerating system was measured as creatine released from phosphocreatine,[11] after appropriate pretreatment of the samples.[12] These data were then used to determine the ATP:2e$^-$ by comparison with the amount of product formed. Ammonia production was determined by the indophenol method[13] after appropriate pretreatment of the samples.[14]

**EPR spectroscopy**. Very low electron-flux conditions, *i.e.*, a 1:100 molar ratio of Fe protein-to-MoFe protein, was used to produce a *ca.* 1:1 equilibrium mixture between the resting-state EPR-active ($E_0$) and the one-electron-reduced EPR-silent ($E_1H$) states as described previously.[15] EPR spectra were recorded on a Varian Associates E-line instrument at a microwave frequency of 9.17 GHz and a microwave power of 10 mW with a 100-kHz field modulation of 25 G at 12K maintained by liquid helium boil-off.

**Stopped-flow spectrophotometry**. Several types of experiments were performed. First, a 1:1 mixture of the $E_0$ and $E_1H$ redox states of the $\alpha277^{Cys}$ MoFe protein was produced as described[15] and used to determine if the 70-ms oxidation involved the 1-electron reduced state ($E_1H$). When

such a mixture is mixed with an eight-fold excess of Fe protein in the presence of excess MgATP and dithionite, a significant increase in the magnitude of the 70-ms oxidation (compared to using $E_0$ is observed.

Second, for the complex-dissociation experiments,[44] oxidized Fe protein (40 μM) is pre-incubated at 23°C with dithionite-free MoFe protein (40 μM) and MgADP (5 mM) in one syringe of the stopped-flow instrument (to form an "artificial" complex) and then rapidly mixed with a solution containing reduced Fe protein, MgADP (5 mM) and dithionite (20 mM) in the other syringe. A series of scans are accumulated at a variety of reduced Fe-protein concentrations (50-250 μM before mixing) to fit the data to a single exponential function. These experiments observe a single complex dissociation. The increasing concentration of reduced Fe protein increases the rate of complex dissociation until the maximum rate is achieved and this is the rate-limiting step. All stopped-flow traces are simultaneously fitted while allowing the rate constants, the absorbance change associated with the reduction of oxidized Fe protein, and the baselines to vary independently.

Third, the FldH$_2$-driven turnover assay was used in attempts to gain insight into the behavior of the more-reduced states of the $\alpha 277^{Cys}$ MoFe protein. The FldH$_2$-oxidation experiment monitors real-time enzyme turnover under dithionite-free conditions. A solution of 1 μM reduced MoFe protein and 8 μM reduced Fe protein was prepared to form a protein-protein complex, which was then mixed with 250 μM FldH$_2$ and 9 mM ATP; both solutions were in 25 mM HEPES buffer (pH 7.4) containing 10 mM MgCl$_2$. The absorbance change at 580 nm was monitored as FldH$_2$ is oxidized to its semiquinone as it reduces the oxidized Fe protein released from the complex. CO was added to the protein solution by gas-tight syringe before loading into the stopped-flow apparatus.

**Table S1**. Diazotrophic Growth Rates and

Crude-Extract MoFe-protein Specific Activities of α192 Mutant Strains.

| Substitution at α192 | Doubling time (h)[a] | MoFe-protein Specific Activity[b] | | | |
|---|---|---|---|---|---|
| | | Under 100% Ar | Under 90% Ar/10% CO | Under 10% $C_2H_2$[c] | |
| | | | | $C_2H_4$ | $H_2$ |
| None (Ser) | 1.8 | 110 | 110 | 105 | 8 |
| Glu | NG | 45 | 105 | 27 | 11 |
| Asp | NG | 40 | 80 | 22 | 15 |
| Thr | 2.2 | 54 | 49 | 27 | 11 |
| Gln | 6.4 | 43 | 42 | 20 | 5 |
| Arg | 10.7 | 4 | 6 | 2 | 1 |
| Trp | NG | 5 | 7 | 2 | 2 |
| Leu | 5.6 | 16 | 16 | 12 | 4 |
| Asn | 3.8 | 22 | 25 | 18 | 5 |

[a] When grown in air on fixed-nitrogen-free Burk medium; NG indicates no growth.

[b] Expressed as nmol $H_2$ or $C_2H_4$ produced per min per mg of total protein under the indicated atmosphere in the presence of excess purified *A. vinelandii* Fe protein.

[c] No $C_2H_6$ is produced by any strain.

**Table S2**. Metal Content and Effect of 10% CO on the $H_2$-evolution Specific Activity and ATP:2e$^-$ Ratio of the Purified Wild-type, $\alpha277^{Cys}$, $\alpha192^{Glu}$ and $\alpha192^{Asp}$ MoFe-protein.

| MoFe protein | | | 100% Ar[a] | | 10% CO/90% Ar[a] | |
|---|---|---|---|---|---|---|
| Type | Mo[b] | Fe:Mo[b] | Specific Activity | ATP:2e$^{-c}$ | Specific Activity | ATP:2e$^{-c}$ |
| Wild Type | 2.0±0.1 | 15:1 | 3000 (690; 345) | 4±0.1 | 2950 (680; 340) | 4±0.1 |
| $\alpha277^{Cys}$ | 1.6±0.1 | 15:1 | 1400 (320; 200) | 10±0.2 | 2400 (550; 345) | 5.5±0.2 |
| $\alpha192^{Asp}$ | 0.8±0.1 | 11:1 | 300 (70; 85) | 24±0.5 | 740 (170; 215) | 10±0.2 |
| $\alpha192^{Glu}$ | 1.4±0.1 | 14:1 | 400 (90; 65) | 16±0.3 | 930 (215; 155) | 6±0.1 |

[a] Expressed as nmol $H_2$ produced per min per mg of purified MoFe protein under the indicated atmosphere in the presence of excess purified *A. vinelandii* Fe protein in 25 mM Tes-KOH at pH 7.4. The parenthetical values are the data converted to nmol $H_2$ per min per nmol MoFe protein and nmol $H_2$ per min per nmol Mo, respectively.

[b] Mo content is expressed as the number of atoms per $\alpha_2\beta_2$ tetramer; Fe content is expressed as the number of Fe atoms per Mo atom.

[c] ATP:2e$^-$ represents the nmol MgATP hydrolyzed per electron pair appearing as product.

**Table S3**. Kinetic Parameters for Purified $\alpha277^{Cys}$, $\alpha192^{Glu}$ and $\alpha192^{Asp}$ MoFe Proteins[a]

| MoFe protein | $K_M$ for $C_2H_2$ $(\%)^b$ | $V_{max}{}^c$ | $K_i$ for CO $(\%)^d$ |
|---|---|---|---|
| Wild Type | 0.6 | 2300 | 0.05 |
| $\alpha277^{Cys}$ | 1.9 | 1240 | 0.40 |
| $\alpha192^{Asp}$ | 1.5 | 240 | 1.10 |
| $\alpha192^{Glu}$ | 1.9 | 275 | 0.70 |

[a] All measurements are made at pH 7.4 in the presence of excess purified *A. vinelandii* Fe protein.

[b] Determined by fitting the data over a $C_2H_2$ concentration range of 0-20% with a standard hyperbolic function using Origin software (Origin, Northampton, MA).

[c] For $C_2H_4$ production only; calculated as described in footnote b.

[d] For CO inhibition of $C_2H_2$ reduction; calculated as described in footnote b, but in the presence of fixed concentrations of CO over the range of 0-10%.

**Figure S1**. Reversibility of CO binding and CO enhancement of $\alpha 277^{Cys}$ MoFe protein $H_2$ evolution. Assays contained 0.25 mg total protein at a 40:1 Fe protein:MoFe protein molar ratio. Time courses for $H_2$ evolution under 100% Ar (●) and 10% CO/90% Ar (○) were run simultaneously. A third time course was run under 10% CO/90% Ar; after 8 min, these vials were evacuated and the experiment continued under 100% Ar (▲).
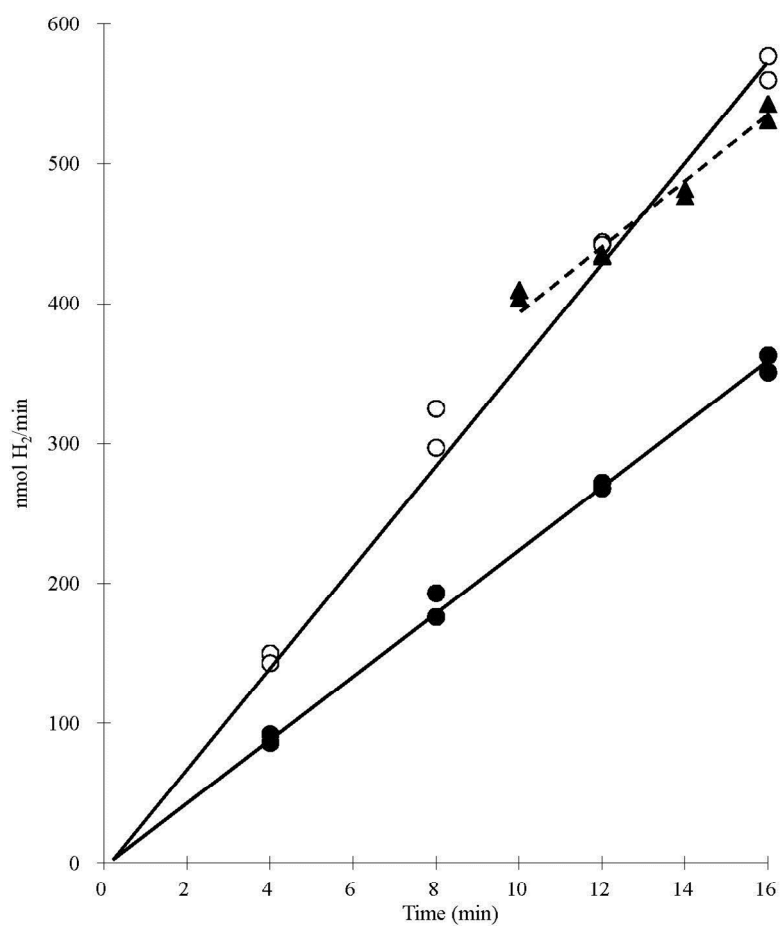
**Figure S2**. Effect of temperature on $H_2$ evolution by $\alpha277^{Cys}$ MoFe protein. The rate of $H_2$ evolution under either 100% Ar (○) or 10% CO/90% Ar (●) was determined over the temperature range of 10-50°C. All assays contained 1 mg total protein at an Fe protein:MoFe protein molar ratio of 40:1. The assay time varied from 4 min at 50°C to 40 min at 10°C.
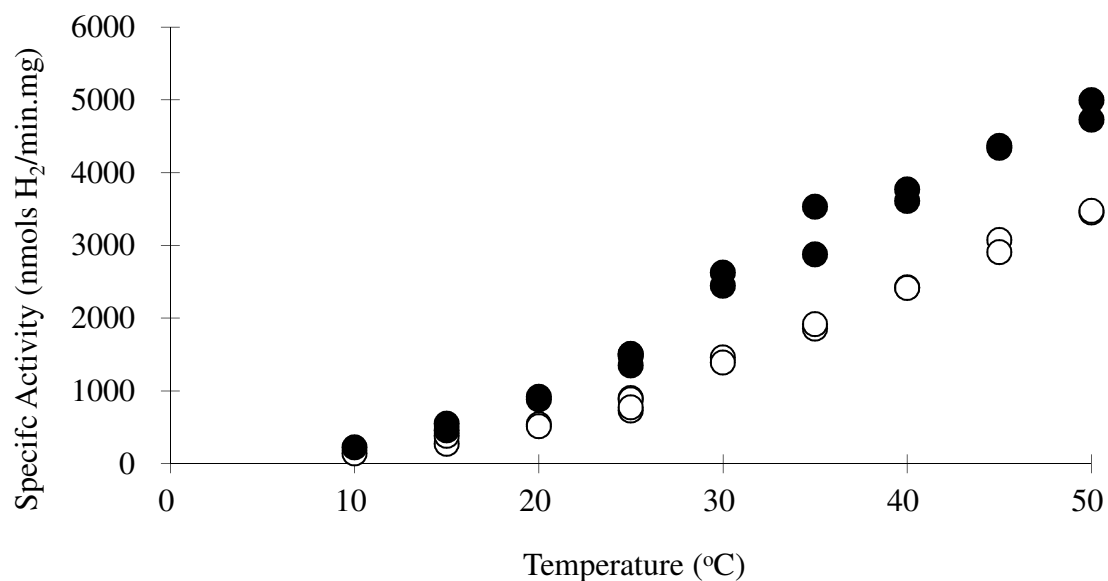
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