Supporting Information

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SI Experimental Procedures

Bacterial Strains and Growth Conditions. Strains used in this work are listed in Table S2. *Azotobacter vinelandii* strains DJ (wild-type) (D.R. Dean, Virginia Tech), DJ1041 (P_{nifH}::*his*$_7$-*nifEN* Δ*nifHDKTYorf1orf2*) (1), and UW243 (2) have been described. Strain UW300 (P_{nifH}::*his*$_9$-*nifQ*) was generated by transforming DJ strain with plasmid pRHB272.

A. vinelandii cells were cultivated in a 150-l fermentor (New Brunswick Scientific BioFlo Pro 150) in 120-l batches of modified Burk's medium with limiting ammonium (80 μg/ml N). Nitrogenase derepression occurs after nitrogen depletion from the medium. Alternatively, 250-ml cultures of *A. vinelandii* cells were grown in 1-liter flasks in nitrogen-replete medium (400 μg/ml N), and nitrogenase was derepressed by collecting the cells, washing them with nitrogen-depleted medium, and resuspending them in the same volume of nitrogen-depleted modified Burk's medium. Growth conditions in the absence or presence of molybdate, *nif* derepression, and cell breakage have been described (3). *Escherichia coli* DH5α and BL21 (pREP-4) strains were grown in Luria–Bertani medium at 37°C with shaking (250 rpm). For growth of *E. coli* on plates, medium solidified with 1.5% agar was used. Antibiotics were used at standard concentrations (4).

Plasmid Construction and Generation of *A. vinelandii* and *E. coli* Strains. Procedures for the transformation of *E. coli* (5) and *A. vinelandii* (6, 7) have been described.

Plasmid pRHB272 contains the *A. vinelandii nifQ* gene PCR-amplified by using oligonucleotides 5′-GCGGCGGGGAACCACATATGGGCAGCGCCGCGGC-3′ and 5′-GCGGCGATGCGGGAGAATTCGGGTCATATCTCTGC-3′ as primers and ligated into the *Nde*I/*Eco*RI sites of pRHB258 for the expression of His-tagged proteins under the control of the *nifH* promoter (8). To facilitate single recombination in the chromosome, pRHB272 includes a 1.1-kb DNA fragment from the chromosomal region downstream of *Avin02530* inserted into the *Hin*dIII site of pRHB258.

For expressing NifQ in *E. coli* cells, the *nifQ* gene was PCR-amplified from the *A. vinelandii* chromosome using primers 5′-GCGGCGGGGAACCACATATGGGCAGCGCCGCGGC-3′ and 5′-GGCCTCTGCCTGCGCGGCCGCGATGCGGGAGAATC-3′ and cloned into the *Nde*I/*Not*I sites of pRHB153 (2) to generate plasmid pRHB270. Plasmid pRHB270 carries a P_{tac}-GST-TEV site-*nifQ* construct.

A. vinelandii strain UW300 was generated by transforming DJ with plasmid pRHB272, followed by selection of Amp^r colonies in solid Burk's medium. The overexpression of NifQ in *A. vinelandii* UW300 strain was tested by immunoblot analysis of UW300 cell-free extracts developed with anti-NifQ antibodies.

Determination of the Native Molecular Weight of *A. vinelandii* NifQ. The native molecular weight of NifQ was determined by gel filtration chromatography conducted anaerobically under a N_2 atmosphere on a Superdex 75 column (GE Healthcare) attached to an AKTA FPLC system (GE Healthcare). The running buffer was 50 mM Tris·HCl (pH 7.5), 300 mM NaCl, and 10% glycerol. A 0.5 ml/min flow rate was used during the chromatography. The column was calibrated for molecular mass determination by using the following protein standards: catalase (M_r = 232.0 kDa) and ribonuclease A (M_r = 13.7 kDa) (GE Healthcare), and the gel filtration standard mix (Bio-Rad) containing a mixture of thyroglobulin, bovine gamma-globulin, chicken ovalbumin, equine myoglobin, and vitamin B_{12}.

Expression and Purification of *A. vinelandii* NifQ from *E. coli* Cells. *A. vinelandii* NifQ purified from recombinant *E. coli* cells was denominated rNifQ. *E. coli* BL21 (pREP-4) cells were transformed with plasmid pRHB270, and overexpression of the GST-rNifQ fusion protein was induced at 30°C by addition of 0.5 mM IPTG to the culture medium. Purification was performed under anoxic conditions at 8°C by using nitrogen-sparged buffers. Induced *E. coli* cells were collected and resuspended in buffer A [10 mM sodium phosphate, 1.8 mM potassium phosphate buffer (pH 7.3), 140 mM NaCl, 2.7 mM KCl, 10% glycerol] and disrupted at 12,000 psi on a French Press. Cell debris was removed by centrifugation at 25,000 × *g* for 40 min twice. The cell-free extracts were loaded onto a 25-ml glutathione (GSH)-Sepharose 4 Fast Flow affinity column (GE Healthcare). The column was washed with 250 ml of buffer A supplemented with 1% Triton X-100, and the GST-rNifQ fusion protein was eluted in 75 ml of buffer E [50 mM Tris·HCl (pH 8.0), 10% glycerol, 20 mM reduced glutathione]. The GST tag was cleaved by the addition of 10 μg of pure TEV protease per mg of GST-rNifQ followed by incubation for 2 h at 30°C. The protein mixture was then subjected to a series of anaerobic chromatographic steps: gel filtration on Sephadex G-25 (to remove glutathione), affinity to GSH-Sepharose (to remove the GST tag), and Co^{2+}-affinity chromatography (to remove the TEV protease). A typical purification procedure yielded 50 mg of rNifQ from 12 g of *E. coli* cell paste. rNifQ was estimated to be >98% pure based on SDS/PAGE analysis. The anaerobic preparations of pure rNifQ were frozen as droplets into liquid nitrogen until use.

Purification of Other Protein Components. The FeMo-co precursor-containing and FeMo-co precursor-deficient forms of NifEN were purified from cells of *A. vinelandii* strains DJ1041 (9) and UW243 (2), respectively. Both NifEN forms were purified under anaerobic conditions by affinity chromatography on a Co^{2+} resin (>98% purity) and stored in liquid nitrogen until use. His-tagged apo-NifDK was purified from cells of *A. vinelandii* DJ1143 as described in ref. 10. NifH was purified as described in ref. 11 with minor modifications. Methods for the purification of FeMo-co (12) and NifB-co (13) have been described.

SDS/PAGE, Anoxic Native-gel Electrophoresis, and Immunoblot Analyses. The procedure for SDS/PAGE has been described (14). Immunoblot analysis was performed as described in ref. 15. Purified preparations of NifQ described in this work were used to raise anti-NifQ antibodies at Capralogics (Hardwick, MA). For anoxic native gel electrophoresis, proteins were separated for 23 h at 100 V on gels with superimposed 7–20% acrylamide and 0–20% sucrose gradients as described in ref. 16. Native gels were then stained for proteins with Coomassie R-250 by standard procedures or stained for iron as described in ref. 17.

Fe and Mo Determinations. The Fe and Mo contents of purified NifQ preparations were routinely determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Concentrated samples of purified NifQ were mixed with 25 mM Tris·HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 1% *N*-methylformamide, and 2% nitric acid for a total volume of 0.5 ml. The mixture was heated at 99°C for 30 min with periodical vortexing to ensure full analyte extraction. The precipitated

protein was pelleted out of the mixture by centrifugation at 18,000 × *g* for 15 min. The supernatant was transferred to a 1.5-ml microfuge tube and introduced into the ICP-OES system (Optima 5 × 00 DV ICP-OES) via a peristaltic pump. The sample was aspirated with humidified argon, passed onto the plasma, and analyzed for Fe and Mo.

Miscellaneous Assays. Protein concentration in the samples was determined by using the bicinchoninic acid method (BCA reagent, Pierce) using Bovine serum albumin as standard (18). Colorimetric Fe determination was performed as described in ref. 19 with minor modifications. UV-visible spectroscopy was carried out in a Shimadzu UV1601V spectrophotometer.

1. Goodwin PJ, *et al*. (1998) The *Azotobacter vinelandii* NifEN complex contains two identical [4Fe-4S] clusters. *Biochemistry* 37:10420–10428.
2. Hernandez JA, *et al*. (2007) NifX and NifEN exchange NifB cofactor and the VK-cluster, a newly isolated intermediate of the iron-molybdenum cofactor biosynthetic pathway. *Mol Microbiol* 63:177–192.
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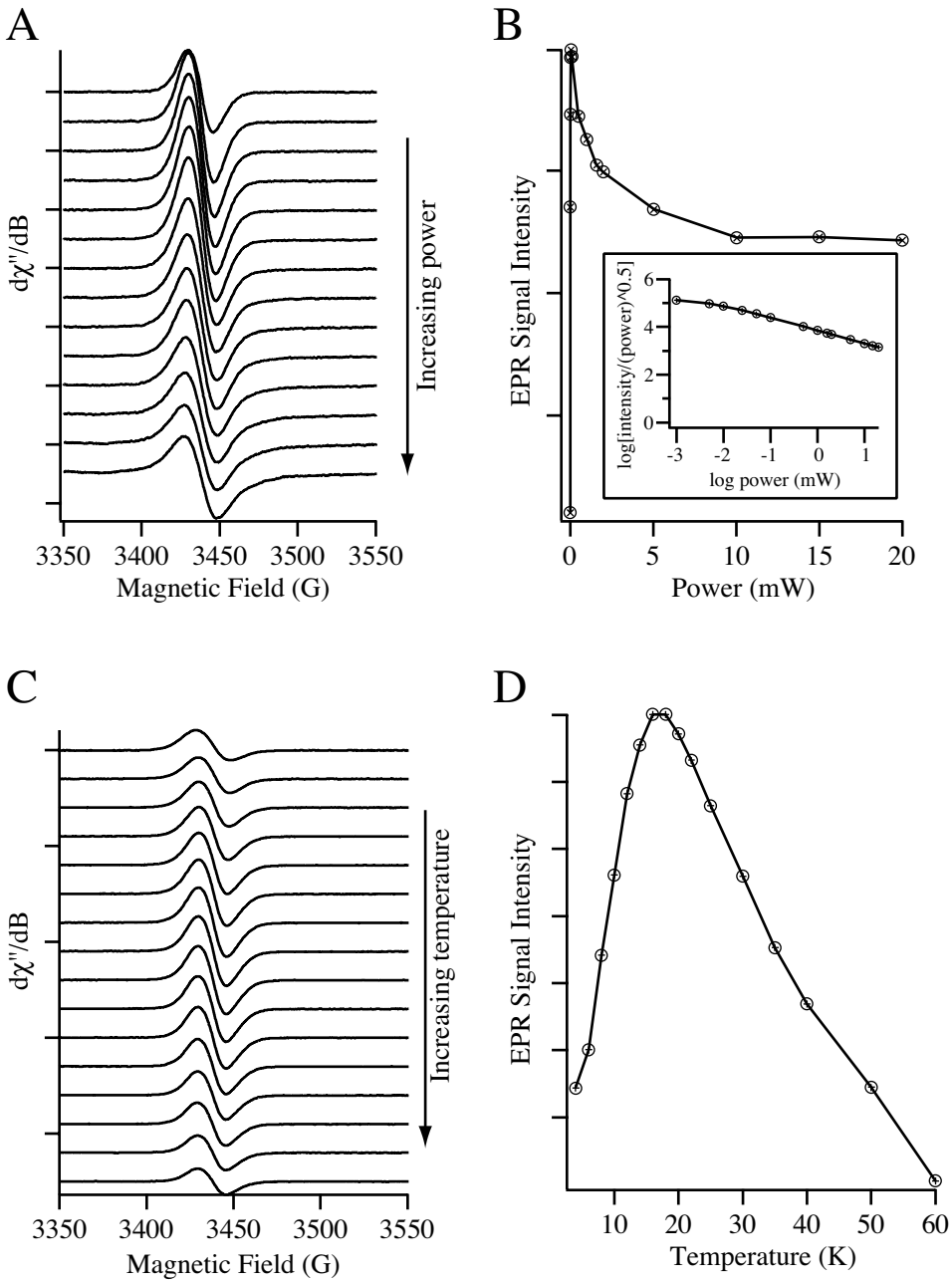

Fig. S1. Power- and temperature dependence profiles of the EPR signal from as-isolated NifQ. Samples contained ≈1 mM NifQ. (*A*) Changes in EPR signal intensity as a function of applied microwave power (0.001–20 mW) at a temperature of 12 K. (*B*) Plot of EPR signal intensity vs. power (mW). (*C*) Changes in EPR signal intensity as function of temperature. The spectra were obtained at a nonsaturating power of 0.032 mW. (*D*) Plot of EPR signal intensity vs. temperature (K). Other experimental conditions are identical to those specified in Fig. 2.

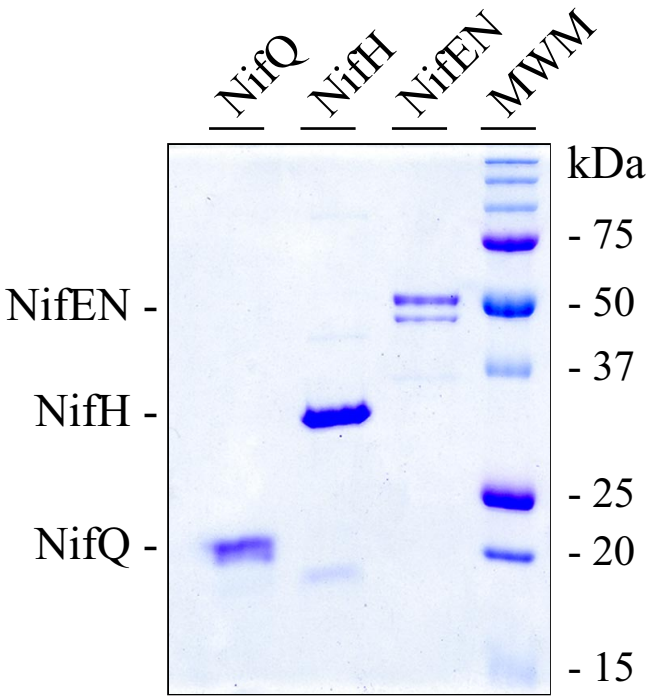

Fig. S2. SDS/PAGE analysis of NifQ, NifH, and NifEN proteins reisolated after incubation in conditions of *in vitro* FeMo-co synthesis. Molecular weight of protein markers are shown to the right.

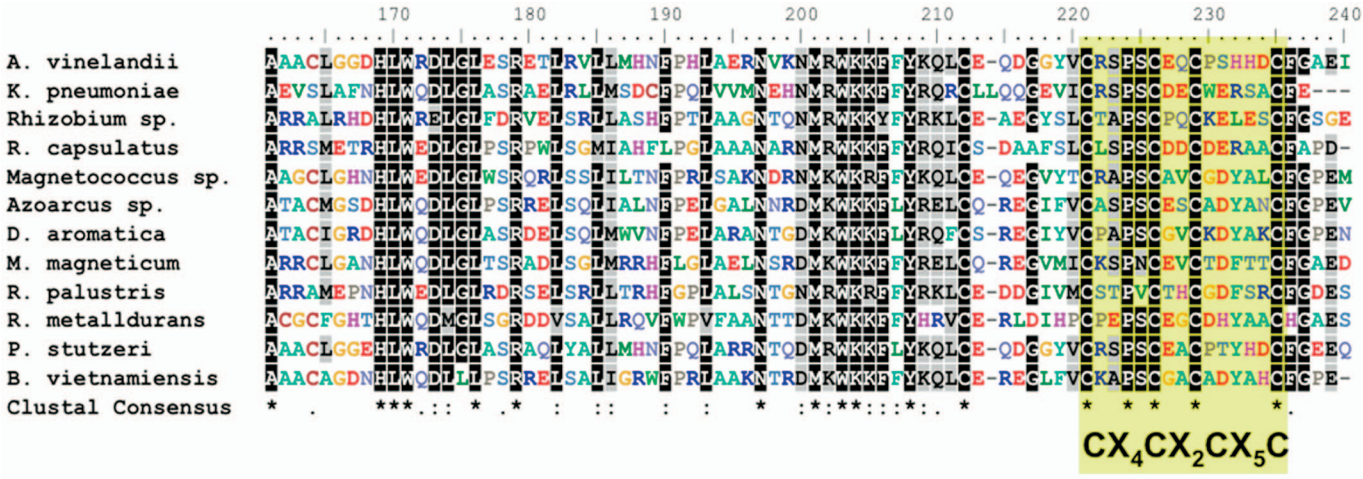

Fig. S3. Amino acid sequence alignment of the C-terminal domain of NifQ proteins from nitrogen-fixing Proteobacteria (*A. vinelandii, Klebsiella pneumoniae*, *Rhizobium* sp., *Rhodobacter capsulatus*, *Magnetococcus* sp., *Azoarcus* sp., *Dechloromonas aromatica*, *Magnetospirillum magneticum*, *Rhodopseudomonas palustris*, *Ralstonia metallidurans*, *Pseudomonas stutzeri*, and *Burkholderia vietnamiensis*). The C-terminal region of NifQ displays a large degree of similarity among nitrogen-fixing Proteobacteria isolated from different environments. Six of the eight cysteine residues of NifQ are located in the C-terminal region. A conserved $Cx_4Cx_2Cx_5C$ motif that suggests [Fe–S]-cluster binding capability is present in this region. Asterisk, conserved residue; semicolon, conservative amino acid substitution; period, nonconservative amino acid substitution.

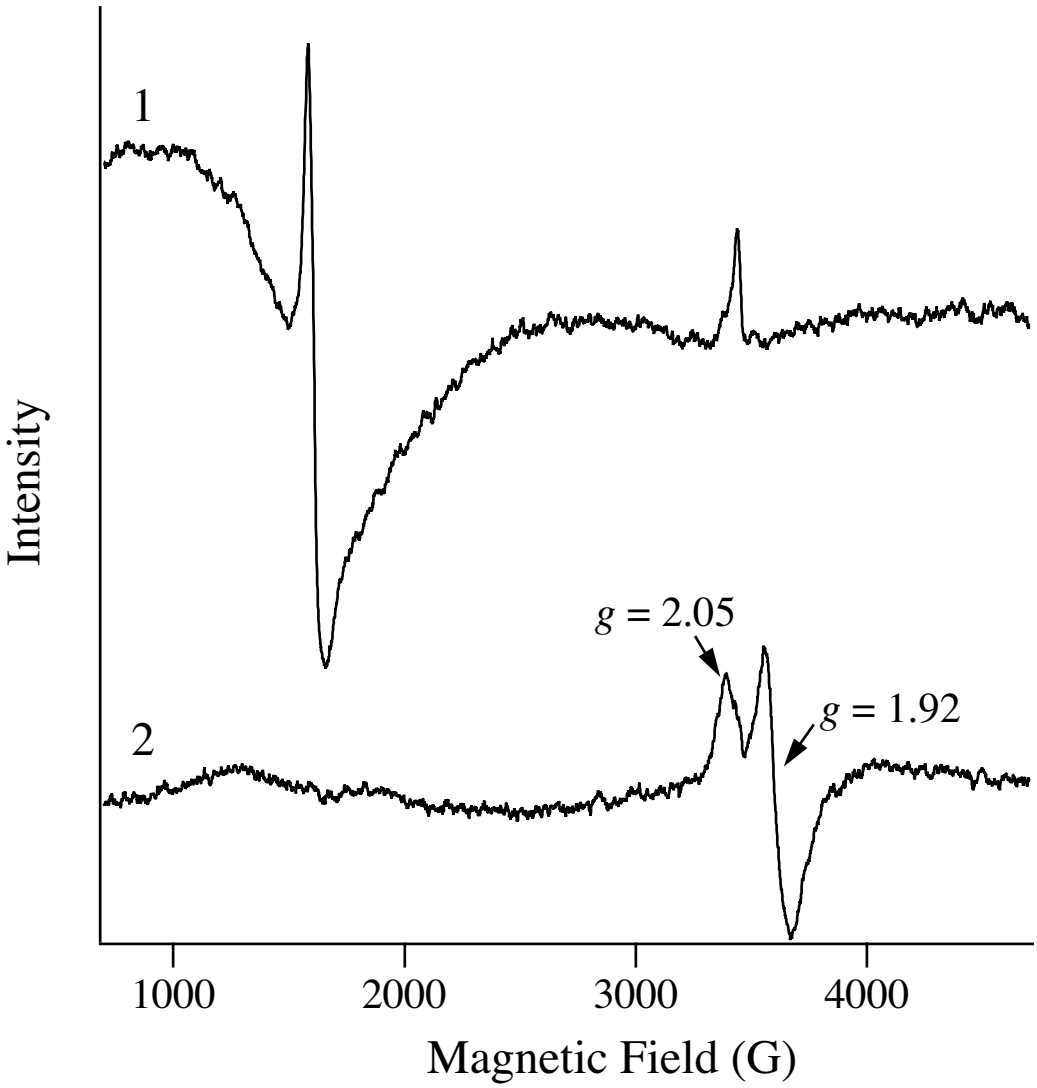

Fig. S4. X-band continuous wave electron paramagnetic resonance analysis of rNifQ. Samples contained ≈1 mM rNifQ. Trace 1, as-isolated rNifQ; trace 2, sodium dithionite (DTH)-reduced rNifQ (5 mM DTH). Spectra were collected with microwave power of 5 mW, microwave frequency of 9.70 GHz, modulation amplitude of 8 G, modulation frequency of 100 kHz, time constant of 80.97 ms, conversion time of 10.24 ms, and resolution of 4096 points.

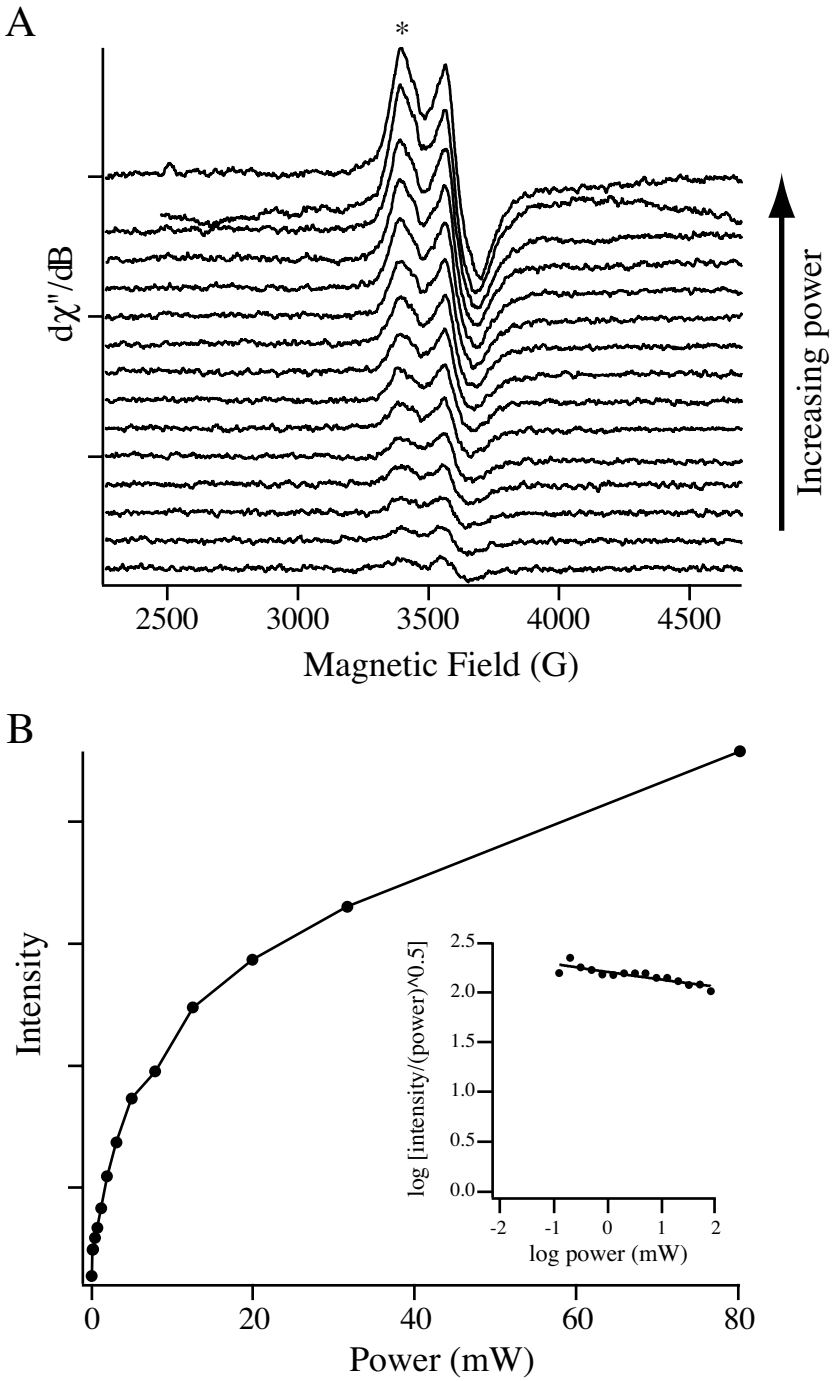

Fig. S5. Power dependence profiles of the EPR signal from DTH-reduced rNifQ. Samples contained ≈1 mM rNifQ. (*A*) Changes in EPR signal intensity as a function of applied microwave power (0.12–80 mW) at a temperature of 12 K. (*B*) Plot of EPR signal intensity vs. power (mW). The asterisks mark the positions in the magnetic field where the EPR signal intensity was measured. Other experimental conditions are identical to those specified in Fig. S4.

Table S1. NifDK activity in *A. vinelandii* cells overexpressing *nifQ*

Strain	Genotype	NifDK activity in the extract	
		Plus NifH	After *in vitro* FeMo-co synthesis
DJ	Wild-type	71.8 ± 1.1	80.8 ± 1.4
UW300	P_{nifH}::his_9-*nifQ*	80.0 ± 5.8	93.8 ± 0.7

Cells were grown in molybdenum-free medium and derepressed for nitrogenase in the presence of 1 μM molybdate. NifDK activity was determined in *A. vinelandii* cell-free extracts after addition of excess NifH by the acetylene reduction assay (1). *In vitro* FeMo-co synthesis and insertion assays were performed as described in *Experimental Procedures*. Reconstituted NifDK activity was assayed by the acetylene reduction assay (1). Activities are expressed in nmol C_2H_4 formed per min per mg protein in the extract. Values are the average of two to four different independent determinations ± SD.

1. Shah VK, Brill WJ (1973) Nitrogenase. IV. Simple method of purification to homogeneity of nitrogenase components from *Azotobacter vinelandii*. *Biochim Biophys Acta* 305:445–454.

Table S2. List of strains and plasmids used in this work

	Genotype and origin	Source or ref.
Strain		
A. vinelandii		
DJ	Wild type	1
UW300	P_{nifH}::*his*$_9$-*nifQ*	This work
DJ1041	Δ*nifHDK-TYorf1orf2,* P_{nifH}::*his*$_7$-*nifEN*	2
UW243	Δ*nifB*:: Kan^R Δ*nifHDK-TYorf1orf2,* P_{nifH}::*his*$_7$-*nifEN*	3
E. coli		
DH5α	F^- ϕ80Δ*lacZM15* Δ(*lacZYA-argF)U169 deoP recA1 endA1 hsdR17* (r_K^- m_K^-)	4
BL21 pREP-4	F^- (*ompT* $r_B^-m_B^-$), Kan^R	Novagene
BL21 pREP-4 (pRHB270)	F^- (*ompT* $r_B^-m_B^-$), Kan^R, P_{tac}::GST-*nifQ*	This work
Plasmids		
pRHB270	pGEX-4T-3-, GST-*nifQ*	This work
pRHB272	pBS, P_{nifH}::*his*$_9$-*nifQ*	This work
pGEX-4T-3	Vector to express GST-fused proteins	GE Healthcare
pBluescript SK	pBS, cloning vector	Fermentas
pGEM-T	Cloning vector	Promega

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