SUPPORTING METHODS

Electron Paramagnetic Resonance Characterization of Three Iron-Sulfur Clusters Present in the Nitrogenase Cofactor Maturase NifB from *Methanocaldococcus infernus*

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MATERIALS AND METHODS

Database analyses and conserved NifB amino acid residues. Forty-two NifB sequences representing a wide diazotrophic diversity were selected for this study (Table S5). ClustalW2 alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) of these sequences revealed a pattern of highly conserved motifs. Importantly, some of these motifs were rich in Cys residues that may serve as ligands to [Fe-S] clusters associated with NifB activity.

Plasmids, strains, and growth conditions. *E. coli* codon-optimized sequences of wild-type *Methanocaldococcus infernus nifB* (encoded by the *metin_0554* gene; http://www.ncbi.nlm.nih.gov/nuccore/CP002009), and site-directed variants substituting Ala for Cys in the N-terminal radical-SAM motif (AXXXAXXA), a C-terminal Cys-rich motif (AXXA), and both motifs (AXXXAXXA/AXXA) were ordered from GenScript and cloned into the *Nde*I and *Xho*I sites of pET16b expression vector to generate plasmids pRHB509, pRHB707, pRHB705, and pRHB706, respectively (Supplemental Figure S8). Plasmid pRSFIscMetKDuet-1 (kindly donated by Juan Fontecilla) is a pRSFDuet-1 (Novagen) derivative generated by inserting *E. coli* K12-MG1655 *isc* gene cluster (lacking the *iscR* gene) into its *Nco*I and *Not*I sites, and the SAM synthetase gene *metK* gene into its *Nde*I and *Xho*I sites.

For overexpression of *Mi*NifB WT and the site-directed variants, *E. coli* BL21 (DE3) carrying pRSFIscMetKDuet-1 was transformed with pRHB509, prHB705, pRHB706 and pRHB707, respectively. Recombinant *E. coli* cells were routinely grown in 50-l batches of Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 4 g/l glycerol adjusted to pH 7.1 with 17 mM KH_2PO_4 and 72 mM K_2HPO_4). The fermentation processes were initially performed under aerobic conditions (sparging 50 l of air per min and shaking at 200 rpm) for around 17 h until an OD_{600} value of 10 was reached. Cultures were then sparged with N_2 and induced for NifB expression for an additional 3 h by adding 3 g/l lactose, 0.2 mM ammonium iron citrate, 2 mM cysteine, and 25 mM di-sodium fumarate. After the fermentation processes, cells (ca. 500 g) were

collected under anaerobic conditions in a GE Hollow Fiber system and sedimented by centrifugation. Solid cell paste was immediately frozen and stored under liquid N_2.

***Mi*NifB purification**. Except when specified as aerobic *Mi*NifB purification, all purification steps were performed at room temperature inside a glovebox as described in[1,2] with modifications. A standard *Mi*NifB anaerobic purification procedure started with 300 g of cells from *E. coli* BL21 (DE3) pRSFIscMetKDuet-1 pRHB509 resuspended for 30 minutes in 900 ml buffer A (50 mM Tris-HCl, pH 8, 200 mM KCl, 30 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, 0.05% n-dodecyl- β -D-maltopyranoside, 0.1% triton X-100 and 0.1% Tween 20 supplemented with lysozyme (0.5 mg/ml), 100 μM phenylmethylsulfonyl fluoride (PMSF), leupeptin (0.5 μg/ml), and DNAse I (5 μg/ml)) inside a glovebox at <2 ppm O_2. Cell-free extract was produced after centrifugation at 70,000 x *g* for 30 minutes. Extract was loaded into a previously equilibrated IMAC HI trap Ni^{2+}-charged column following manufacturer's instructions. The column was washed with 5 column volumes (CV) of buffer B (50 mM Tris-HCl, pH 8, 200 mM KCl, 30 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol) and 2 CV of buffer C (50 mM Tris-HCl, pH 8, 200 mM KCl, 100 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol). Elution was performed with 1-2 CV of buffer D (50 mM Tris-HCl, pH 8, 200 mM KCl, 250 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol). Elution fractions were analyzed by SDS-PAGE. Valid eluted fractions, containing pure *Mi*NifB, were pooled and desalted in a GE HiPrep 26/10 desalting column using buffer E (50 mM Tris-HCl, pH 8, 200 mM KCl, 10% glycerol, 5 mM β-mercaptoethanol). The final sample was concentrated in 3 kDa centricons and stored in liquid N_2 drops.

Generation of apo-*Mi*NifB and *in vitro Mi*NifB [Fe-S] cluster reconstitution. To remove [Fe-S] clusters and generate apo-*Mi*NifB, purified enzyme preparations were treated with 10 mM

EDTA under reducing conditions in 5 mM DTH as described previously.[3,4] **[**Fe-S] cluster reconstitutions were performed under anaerobic conditions in Buffer A (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 10% Glycerol, 5 mM β-Mercaptoethanol). *Mi*NifB samples (10 μM) were incubated during 2 h at 50ºC with a 12-fold molar excess of Fe^{2+} ($(NH_4)_2Fe(SO_4)_2$) and S^{2-} (Na_2S) in the presence of 10 mM DTT. After the incubation, the Fe and S excess was removed by filtration through a HiPrep 26/10 Desalting column (GE Healthcare life Science) previously equilibrated with Buffer A.

***Mi*NifB activity assays.** *Mi*NifB activity was determined under strict anaerobic conditions inside an M'Braun glovebox (O_2 < 0.1 ppm) by a coupled assay that included *in vitro* FeMo-co synthesis and apo-NifDK activation as described in[5] with modifications. First, 80 μM *Mi*NifB was incubated for 30 min at 60ºC in 0.2 ml of a reaction mixture containing 5 mM DTH, 1 mM SAM, 500 μM $FeSO_4$, and 500 μM Na_2S. Control reactions substituted NifB-co[6] for *Mi*NifB. Fifty μl aliquots of *Mi*NifB were then transferred to reaction mixtures for *in vitro* FeMo-co synthesis and apo-NifDK activation and incubated for 30 min at 30ºC. Complete reaction mixtures (0.4 ml) contained: 22 mM Tris-HCl, pH 7.4, 3.5% glycerol, 17.5 μM Na_2MoO_4, 175 μM *R*-homocitrate, 10 μM *Mi*NifB, 3 μM NifX, 1.2 μM NafY, 3.5 μM apo-NifEN, 3.0 μM NifH, and 0.6 μM apo-NifDK. Activity of reconstituted NifDK was analyzed by the acetylene reduction assay after addition of excess NifH and 0.42 ml of ATP-regenerating mixture (1.32 mM ATP, 18 mM phosphocreatine, 2.2 mM $MgCl_2$, 3 mM DTH and 40 μg creatine phosphokinase). Ethylene formation was measured in a Shimadzu GC-2014 gas chromatograph equipped with a Porapak N 80/100 column.[7]

For time-course experiments with simultaneous detection of ethylene, SAH and AdoH, the 60˚C *Mi*NifB pre-incubation phase was omitted. *Mi*NifB-dependent FeMo-co synthesis and apo-NifDK

activation reactions were performed at 40˚C in a series of 0.8 ml reaction mixtures as described above. Reactions were split into 0.4 ml aliquots at different times (0, 15, 30, 45 and 60 min), stopped, and processed differently. In aliquots used to determine NifDK reconstitution, reactions were stopped by addition of 0.3 mM $(NH_4)_2MoS_4$ and the acetylene reduction assay performed after addition of excess NifH and ATP-regenerating mixture.[8] In aliquots used to quantify SAH and AdoH, reactions were stopped by addition of 50 µl of 1 M TFA and exposure to air, and then analyzed by High Pressure Liquid Chromatography (HPLC). Reaction samples (0.25 ml) were injected onto a Zorbax SB-C18 column (protected with a pre-guard SB-C18 column) equilibrated with 0.1% TFA and resolved with a 0-100% linear gradient of acetonitrile in 0.1% TFA at 1 ml/min for 25 min. AdoH and SAH were identified by UPLC/ESI-qTOF-MS by comparing their profiles to those of commercial standards (Sigma-Aldrich D1771 and A9384, respectively) as described below.

UPLC/ESI-qTOF-MS. Dried samples were re-suspended in 50 µl acidified methanol (methanol, 0.1% formic acid [v/v]) Five microliters of the solution were separated using a Dionex UltiMate3000 RSLC system (Dionex, Sunnyvale, USA). The column used was a 50 mm x 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH C18 column with a 5 mm x 2.1 mm i.d., 1.7 µm, VanGuard pre-column of the same material, and a 0.2 µm in-line filter set (Waters, Milford, USA). The following binary gradient was applied: 0 to 1 min isocratic 100% A (deionized water, 0.1% formic acid [v/v]); 1 to 10 min linear gradient to 28% B (acetonitrile, 0.1% formic acid [v/v]); 10 to 15 min linear gradient to 100% B; then 100% B isocratic for 2 min; re-conditioning of the column, 2.5 min isocratic 100% A. The flow rate was 400 µl/min. The autoinjector temperature was set to 4 ºC, the column compartment was maintained at 30 ºC.

Eluted compounds were detected by a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization source in positive ion mode. Typical

instrument settings were as follows: capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 200 ºC; dry gas flow, 10 l/min. Ions were detected from m/z 50 to 1000 at a repetition rate of 2 Hz. Mass calibration was performed using sodium formiate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

^{15}N-SAM preparation. ^{15}N-SAM was synthesized starting with ^{15}N-methionine and purified as described.[9]

Deuterium substitution experiments. Each reaction contained, in a total volume of 150 µl, 25 mM Tris-HCl (pH 7.4), 10% glycerol, 500 µM β-Mercaptoethanol, 5 mM DTH and 10 µM *Mi*NifB. The reaction was initiated by the addition of 5 mM unlabeled SAM (Sigma-Aldrich) or 500 µM $^{13}CD_3$-SAM incubated for 15 min at 65°C with intermittent mixing, and subsequently terminated by removing *Mi*NifB from the solutions using Amicon Ultra 30,000 MW centrifugal filters. Filtered samples were then analyzed by HPLC and LC-MS at CBGP metabolomic facility. SAM was synthesized starting with unlabeled methionine or with labeled $^{13}CD_3$-methionie and purified as described.[9] Synthesized $^{13}CD_3$-SAM and ^{15}N-SAM were kindly donated by Dr. Etienne Mulliez.

Protein methods. Protein concentration was determined by bicinchoninic acid method (Pierce), with bovine serum albumin as the standard.[10] Colorimetric Fe and S determination were performed according to[11] and [12], respectively. UV visible absorption spectra were recorded under anaerobic conditions in septum-sealed cuvettes inside M'Braun glove box using a Shimadzu UV-2600 spectrophotometer equipped with optical fibers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard methods. Protein mass spectrometry

was carried out with a 4800 Proteomics Analyzer equipped with TOF/TOF mass spectrometer at the Proteomics Unit of Parque Científico de Madrid.

Electron Paramagnetic Resonance (EPR) Spectroscopy. Samples were prepared in a Vacuum Atmospheres Company (VAC, model LM02) nitrogen atmosphere anaerobic glove box by mixing the reaction components and filling a 4.0 mm O.D. x 10 cm quartz tube, sealed with rubber septa, and freezing in liquid nitrogen or an acetone/dry ice bath immediately upon removal from the glove box. The EPR spectra were measured at the CalEPR Center at the University of California, Davis. Continuous-wave (CW) X-band spectra were acquired with a Brüker Elexsys-II E500 spectrometer (Brüker, Billerica, MA) under non-saturating conditions using a Super-High Q resonator (ER 4122SHQE). Cryogenic temperatures were achieved and maintained using an Oxford Instruments ESR900 liquid helium cryostat in conjunction with an Oxford Instruments ITC503 temperature and gas flow controller. Unless indicated, CW spectra were measured at 9.38 GHz microwave frequency, 1 mW power, 5 G modulation amplitude, at 15 K. Three pulse electron spin echo envelope modulation (3P-ESEEM) data were acquired with a Brüker EleXsys E580 pulse EPR spectrometer equipped with an Oxford-CF935 liquid helium cryostat and an ITC-503 temperature controller using the pulse sequence $\pi/2$-τ-$\pi/2$-T-$\pi/2$-echo where the delay time, T, is varied. 3P-ESEEM were collected at 10 K, τ = 128 ns, $\pi/2$ = 12 ns, and a magnetic field of 340 mT. Data processing and spectra simulations were performed with Matlab using the EasySpin 4.0 toolbox.[13]

Spin Counting. Concentrations of EPR samples of NifB are calculated using a 300 μM Cu(II)-EDTA standard collected at 100K, 1 mW microwave power, and 3G modulation amplitude. Spectra intensities were quantified by taking the double integral of the collected data and adjusting for temperature, power and modulation amplitude by the equation:

$I = I_{rel}*T/(sqrt(P)*A)$

Where I is the parameter adjusted intensity, I_{rel} is the unadjusted maximum of the double integral of the spectrum, T is temperature in kelvin, P is the microwave power and A is the modulation amplitude.

References

(1) Echavarri-Erasun, C.; Arragain, S.; Rubio, L. M. *Methods Mol. Biol.* **2014**, *1122*, 5-18.

(2) Echavarri-Erasun, C.; Arragain, S.; Scandurra, A. A.; Rubio, L. M. *Methods Mol. Biol.* **2014**, *1122*, 19-31.

(3) Arragain, S.; Garcia-Serres, R.; Blondin, G.; Douki, T.; Clemancey, M.; Latour, J. M.; Forouhar, F.; Neely, H.; Montelione, G. T.; Hunt, J. F.; Mulliez, E.; Fontecave, M.; Atta, M. *J. Biol. Chem.* **2010**, *285*, 5792-5801.

(4) Pierrel, F.; Hernandez, H. L.; Johnson, M. K.; Fontecave, M.; Atta, M. *J. Biol. Chem.* **2003**, *278*, 29515-29524.

(5) Curatti, L.; Hernandez, J. A.; Igarashi, R. Y.; Soboh, B.; Zhao, D.; Rubio, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17626-17631.

(6) Shah, V. K.; Allen, J. R.; Spangler, N. J.; Ludden, P. W. *J. Biol. Chem.* **1994**, *269*, 1154-1158.

(7) Shah, V. K.; Brill, W. J. *Biochim. Biophys. Acta* **1973**, *305*, 445-454.

(8) Shah, V. K.; Ugalde, R. A.; Imperial, J.; Brill, W. J. *J. Biol. Chem.* **1985**, *260*, 3891-3894.

(9) Iwig, D. F.; Booker, S. J. *Biochemistry* **2004**, *43*, 13496-13509.

(10) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76-85.

(11) Fish, W. W. *Methods Enzymol.* **1988**, *158*, 357-364.

(12) Beinert, H. *Anal. Biochem.* **1983**, *131*, 373-378.

(13) Stoll, S.; Schweiger, A. *J. Magn. Reson.* **2006**, *178*, 42-55.