Supplementary Materials for

Radical SAM-Dependent Carbon Insertion into the Nitrogenase M-Cluster

Jared A. Wiig, Yilin Hu,* Chi Chung Lee, Markus W. Ribbe*

*To whom correspondence should be addressed.
E-mail: yilinh@uci.edu (Y.H.); mriebbe@uci.edu (M.W.R.)

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**Materials and Methods**

Unless noted otherwise, all chemicals and reagents were obtained from Fisher Scientific or Sigma-Aldrich.

**Cell Growth and Protein Purification.** All *A. vinelandii* strains were grown in 180 L batches in a 200 L New Brunswick fermentor (New Brunswick Scientific) in Burke’s minimal medium supplemented with 2 mM ammonium acetate. The growth rate was measured by cell density at 436 nm using a Spectronic 20 Genesys spectrophotometer (Spectronic Instruments). Following ammonia depletion, cells were de-repressed for 3 hr and then harvested immediately using a flow-through centrifugal harvester (Cepa). The cell paste was washed with a 50 mM Tris-HCl (pH 8.0) buffer. Published methods were adapted to the purification procedures of His-tagged NifEN-B, apo NifEN, apo NifDK (8, 13, 32) and the non-tagged apo NifDK and NifH (33).

**SAM Cleavage Assay.** The SAM cleavage assay contained, in a total volume of 1 mL, 25 mM Tris-HCl (pH 8.0), 20 mM sodium dithionite (Na$_2$S$_2$O$_4$), 5% glycerol, 10 µM SAM, and 16 µM NifEN-B. These assays were incubated at 25°C for 30 min with intermittent mixing and terminated by removing NifEN-B from the solutions using an Amicon Ultra 30,000 MWCO centrifugal filter. The filtered samples were then analyzed by an Agilent 1200 Series HPLC system (Santa Clara, CA) on a Supelcosil LC-18-T HPLC column (15 cm x 4.6 mm, 5 µm particle size). 100 µL of each sample was injected onto this column, which was equilibrated with buffer A (2% methanol, 50 mM KH$_2$PO$_4$, pH 6.6). Then, a gradient of 0-75% buffer B (80% methanol) was applied to the column at a rate of 0.5 mL/min for 20 min, followed by an isocratic flow of 75% buffer B for 10
min and 0% buffer B for another 5 min at the same flow rate. The column was then equilibrated by 0% buffer B for 10 min before the next sample injection. Elution of SAM cleavage products was monitored by continuous UV/vis detection at 254 nm, and data analysis was performed by using Agilent ChemStation software.

**LC-MS Analysis.** LC-MS analysis was performed on an Agilent 1100 Series HPLC system with a Gilson 231 XL autosampler (10 µL injections) coupled to a Waters Micromass LCT mass spectrometer. Products were separated on a Phenomenex Kinetex C18 column (100 mm x 2.1 mm, 2.6 µm particle size) that was equilibrated in 98% solvent A (97.9% H2O, 2% methanol, 0.1% acetic acid) and 2% solvent B (99.9% methanol, 0.1% acetic acid). A gradient of 2-95% solvent B was applied over 50 min at 0.2 mL/min, followed by an isocratic flow of 95% solvent B for 7 min at the same flow rate. The column was prepared for subsequent sample injection by applying a gradient of 95-2% solvent B, followed by an equilibration period of 5 min at 2% solvent B. Product detection was performed via electrospray ionization in positive ion mode (ESI⁺) with the following mass spectrometer parameters: capillary voltage, 3,000 V; sample cone voltage, 30 V; desolvation temperature, 120 °C; and source temperature, 120 °C. In order to identify the daughter fragments of the SAM cleavage products, the sample cone voltage was increased to 80 V, which resulted in the same fragmentation patterns as those of the SAH and 5’-dAH controls, respectively. MassLynx software was used for data collection and analysis.

**Deuterium Substitution Experiments.** Each reaction contained, in a total volume of 150 µL, 25 mM Tris-HCl (pH 8.0), 20 mM Na2S2O4, 5% glycerol, and 140 µM NifEN-B. These reactions were
initiated by the addition of 330 μM unlabeled SAM (Sigma-Aldrich) or 330 μM [methyl-$d_3$] SAM (C/D/N Isotopes Inc.), incubated for 30 min at 25°C with intermittent mixing, and subsequently terminated by removing NifEN-B from the solutions using Amicon Ultra 30,000 MWCO centrifugal filters. The filtered samples were then analyzed by HPLC and LC-MS as described above.

**Carbon-14 Tracing Experiments.** Four different reactions were assembled, each containing, in a total volume of 32 μL, 100 mM Tris-HCl (pH 8.0), 20 mM Na$_2$S$_2$O$_4$, 250 μM [methyl-$^{14}$C] or [carboxyl-$^{14}$C] SAM (American Radiolabeled Chemicals, Inc.) and (1) 10 μM His-tagged NifEN-B alone; (2) 10 μM His-tagged NifEN-B, 12 μM His-tagged apo NifDK, and 26 μM non-tagged NifH; (3) 10 μM His-tagged NifEN-B, 12 μM non-tagged apo NifDK, and 26 μM non-tagged NifH; or (4) 10 μM His-tagged NifEN-B, 12 μM non-tagged apo NifDK, and 26 μM His-tagged NifH. Reactions 2-4 also contained 0.8 mM ATP, 1.6 mM MgCl$_2$, 10 mM creatine phosphate, 8 units creatine phosphokinase, 0.3 mM homocitrate, and 0.3 mM sodium molybdate (Na$_2$MoO$_4$). All reactions were incubated for 20 min at 25 °C with intermittent mixing and then run over IMAC sepharose resin (10 μL packed volume; GE Healthcare) that was equilibrated with a buffer containing 25 mM Tris-HCl (pH 8.0), 2 mM Na$_2$S$_2$O$_4$, 10% glycerol, and 500 mM NaCl. The IMAC sepharose resin was washed 3 times with 700 μL buffer containing 25 mM Tris-HCl (pH 8.0), 2 mM Na$_2$S$_2$O$_4$, 10% glycerol, 500 mM NaCl, and 40 mM imidazole. The flow-thru of IMAC sepharose resin was diluted 4-fold with a buffer containing 25 mM Tris-HCl (pH 8.0) and 2 mM Na$_2$S$_2$O$_4$ and then run over DEAE cellulose resin (20 μL packed volume; GE Healthcare), which was equilibrated with same buffer. The DEAE cellulose resin was then washed 3 times with 700 μL
buffer as described above. The IMAC sepharose and DEAE cellulose samples were then resuspended in the wash buffer and applied directly onto Whatman 3 MM chromatography paper. The blots were dried and exposed to a GE Healthcare Storage Phosphor Screen GP (20 x 25 cm) for 24 to 48 hrs before imaging was performed on a GE Healthcare Typhoon Trio+ variable mode imager.

**SDS-PAGE Analysis.** Protein samples captured by the IMAC sepharose resin (see “Carbon-14 Tracing Experiments” above) were eluted with a buffer containing 25 mM Tris-HCl (pH 8.0), 2 mM Na₂S₂O₄, 10% glycerol, 500 mM NaCl, and 250 mM imidazole, loaded on a 4-20% Mini-PROTEAN TGX precast gel (BioRad), and run at 100 volts in an SDS gel running buffer for 1 hr. An identical gel was prepared, which included an additional control with [methyl-¹⁴C]-SAM cross-linked to the His-tagged NifEN-B by UV treatment. The UV cross-linking procedure was performed as described earlier (34). After electrophoresis, the first TGX gel was stained and destained for direct visualization of polypeptides; whereas the second was fixed with a 45% methanol/10% acetic acid solution, dried on a Hoefer Scientific slab gel drier, and exposed to a GE Healthcare storage phosphor screen GP (20 x 25 cm) for 48 hrs before imaging on a GE Healthcare Typhoon Trio+ variable mode imager.

**Cluster Extraction.** Protein samples captured by the IMAC sepharose or DEAE cellulose resins (see “Carbon-14 Tracing Experiments” above) were eluted with a buffer containing 25 mM Tris-HCl (pH 8.0), 2 mM Na₂S₂O₄, 10% glycerol, 500 mM NaCl, and 250 mM imidazole. The transient cluster species in these protein samples were extracted in small scale by a previously described
procedure (11, 35, 36), which started by denaturing the protein samples in citric acid, followed by pelleting the cluster-bound mass, washing it with dimethylformamide (DMF) and eluting clusters into N-methylformamide (NMF). The extracted cluster species were then blotted onto Whatman 3 MM chromatography paper and examined for radioactivity. In NMF, the extracted clusters retained 99% activity after 24 hrs at room temperature (36).

**Activity Analysis.** Activities of cluster species extracted from NifEN-B (L-cluster) and NifDK (M-cluster) were determined by maturation and reconstitution assays, respectively. The maturation assay contained, in a total volume of 1 mL, 25 mM Tris-HCl (pH 8.0), 20 mM Na$_2$S$_2$O$_4$, 10 µM apo NifEN, 16 µL extracted L-cluster, 23 µM NifH, 0.8 mM ATP, 1.6 mM MgCl$_2$, 10 mM creatine phosphate, 8 units creatine phosphokinase, 0.3 mM Na$_2$MoO$_4$, 0.3 mM homocitrate, and 1 µM apo NifDK. The reconstitution assay contained, in a total volume of 1 mL, 25 mM Tris-HCl (pH 8.0), 20 mM Na$_2$S$_2$O$_4$, 1 µM apo NifDK, and 32 µL extracted M-cluster. For both assays, the reaction mixture was incubated for 30 min at 30°C before it was examined for C$_2$H$_2$-reducing activities (13) and the maximum activities were achieved by titrating activities versus increasing amounts of extracted clusters (11).
**Figures S1**

**Fig. S1.** LC-MS analysis of SAH (A) and 5’-dAH (B). Upon incubation with NifEN-B and reductant, SAM is cleaved into two products (blue), which behave like SAH and 5’-dAH standards (black), respectively, in LC-MS analysis. SAH has a mass-to-charge (m/z) ratio of 385 (A, 1) and displays a fragmentation pattern of the following m/z ratios: 88 (A, 2), 119 (A, 3) and 136 (A, 4); whereas 5’-dAH has an m/z ratio of 252 (B, 1) and displays a fragmentation pattern of the following m/z ratios: 119 (B, 3) and 136 (B, 4).

**Figures S2**

**Fig. S2.** Methylation of rRNA by RlmN or Cfr involves transfer of a methyl group from one equivalent of SAM to a cysteine residue via an SN2 mechanism, followed by generation of a 5’-dA• via reductive cleavage of a second equivalent of SAM, which abstracts a hydrogen atom from the methylcysteine. The resultant methylene radical then initiates the radical-based methylation of rRNA.
**Figures S3**

**Fig. S3.** SDS-PAGE analysis of protein fractions captured by IMAC (A) and DEAE (B) resins. Lane 1, molecular weight marker; lane 2, IMAC (A) or DEAE (B) fraction from Fig. 3A, 1; lane 3, IMAC (A) or DEAE (B) fraction from Fig. 3A, 2; lane 4, IMAC (A) or DEAE (B) fraction from Fig. 3A, 3; and lane 5, IMAC (A) or DEAE (B) fraction Fig. 3A, 4. Also see labels in Fig. 3A for the composition of protein(s) in each IMAC or DEAE fraction.

**Figures S4**

**Fig. S4.** Autoradiogram of [methyl-\(^{14}\)C]-SAM-treated protein samples after SDS-PAGE analysis. The following samples were loaded on the gel after incubation with [methyl-\(^{14}\)C]-SAM: lane 1, His-tagged NifEN-B alone; lane 2, His-tagged NifEN-B, non-tagged NifH, and His-tagged NifDK; lane 3, His-tagged NifEN-B, non-tagged NifH, and non-tagged NifDK; lane 4, His-tagged NifEN-B, His-tagged NifH, and non-tagged NifDK; and lane 5, [methyl-\(^{14}\)C]-SAM cross-linked with NifEN-B. Samples 2-4 also contained dithionite, ATP, molybdate, and homocitrate.
References


9. The reduced K cluster shows an $S = 1/2$ signal that is characteristic of $[\text{Fe}_4\text{S}_4]^+$ clusters, whereas the oxidized L cluster shows a $g = 1.92$ signal that is unique to the $[\text{Fe}_8\text{S}_9]$ precursor of M cluster (8). The assignment of K cluster as a pair of $[\text{Fe}_4\text{S}_4]$ clusters was based on metal determination and quantitative EPR and UV/vis analyses (8), whereas the determination of L cluster as a $[\text{Fe}_8\text{S}_9]$ homolog to the M cluster was based on x-ray absorption spectroscopy, extended x-ray absorption fine structure, and x-ray crystallographic studies (10–12). The conversion of K to L to M cluster on NifEN-B was monitored by activity and EPR analyses (8).


20. Materials and methods are available as supplementary materials on *Science* Online.

21. An alternative mechanism was proposed for RNA methylation by RlmN, which involves hydrogen atom abstraction from the methyl group of SAM by 5′-dA•, followed by transfer of the resultant methylene radical to an amidine carbon of the substrate (16).


26. PTM analyses were performed as described previously (18) at the Biomolecular Mass Spectrometry Facility of University of California–San Diego and the Mass Spectrometry Facility of University of California–Irvine.


