Supplementary Information

Tracing the Incorporation of the “9th Sulfur” into the Nitrogenase Cofactor Precursor with Selenite and Tellurite

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Supplementary Methods

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA). \([\text{PPh}_4\text{][Fe}_4\text{S}_4(\text{SCH}_2\text{CH}_2\text{OH})_4]\) was prepared as described elsewhere.\(^1\) 2-Mercaptoethanol (BME) was degassed by three freeze-pump-thaw cycles prior to use. For biochemical experiments, all manipulations were performed under an Ar atmosphere using Schlenk techniques and a glove box operating at <3 ppm O\(_2\). Aqueous solutions of europium(II) ethyleneglycol- bis(2-aminomethylene)-\(N,N,N',N'\)-tetraacetic acid (Eu\(^{II}\)-EGTA) were prepared immediately before use as described earlier.\(^2\)

Cell Growth, Protein Purification, and Reconstitution. An *Escherichia coli* strain (YM114EE) expressing His-tagged NifB of *Methanosarcina acetivorans* (*Ma*NifB) was grown and induced for protein expression by IPTG as described earlier,\(^3\) followed by purification of *Ma*NifB using published methods.\(^3\) *Azotobacter vinelandii* strains YM9A, DJ1143, and DJ1141, which express His-tagged \(\Delta\text{nifB} \) NifEN (NifEN\(^{apo}\)), His-tagged \(\Delta\text{nifB} \) NifDK (NifDK\(^{apo}\)) and His-tagged wildtype NifDK (NifDK\(^{holo}\)), respectively, were grown and harvested as described previously,\(^4-7\) followed by purification with Ni-affinity chromatography.\(^4-6\) Non-tagged, wildtype NifH was purified from the flow-through of the Ni-affinity column used for the purification of NifDK\(^{holo}\) as described elsewhere.\(^7\) Published methods were used to prepare dithionite (DT)-free *Ma*NifB reconstituted with synthetic [Fe\(_4\)S\(_4\)] clusters (\([\text{PPh}_4\text{][Fe}_4\text{S}_4(\text{SCH}_2\text{CH}_2\text{OH})_4]\)).\(^8\)

Electron Paramagnetic Resonance (EPR) Spectroscopy. The EPR samples of DT-free *Ma*NifB were prepared in a glove box under the Ar atmosphere, frozen in liquid nitrogen, and stored in a liquid nitrogen dewar until use. The DT-free *Ma*NifB samples were incubated at \(\sim26^\circ\)C for 30 min in a buffer containing 2 mM Eu\(^{II}\)-EGTA, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol and (1) no additive; (2) 10 mM SAM; (3) 10 mM SAM and 2 mM Na\(_2\)SO\(_3\); (4) 10 mM SAM and 0.5 mM Na\(_2\)SeO\(_3\); and (5) 10 mM SAM and 0.5 mM Na\(_2\)TeO\(_3\). Excess reductant (Eu\(^{II}\)-EGTA) in these samples was then removed by a Sephadex G-25 desalting column. Alternatively, the samples were incubated with excess oxidant, indigo disulfonate (IDS) for 5 min, followed by removal of excess IDS by a Sephadex G-25 desalting column. The final concentrations of reduced or oxidized *Ma*NifB samples were 250-500 µM.

EPR spectra were recorded by an ESP 300 spectrophotometer (Bruker) equipped with an ESR-900 liquid-helium continuous-flow cryostat (Oxford Instruments) using a microwave power of 1-10 mW, a gain of \(5\times10^4\), a modulation frequency of 100 kHz, and a modulation amplitude of 5 G. Four scans were recorded for each EPR sample at a temperature of 10 K (for IDS-oxidized
samples) or 20 K (for Eu\textsuperscript{II}-EGTA-reduced samples) and a microwave frequency of 9.62 GHz. Pulse EPR studies were performed at the UC Davis CalEPR center, using a Bruker EleXsys E580 pulse EPR spectrometer equipped with an Oxford-CF935 liquid helium cryostat and an ITC-503 temperature controller. Pulse EPR data were collected using a Bruker MS5 probe. Three-pulse ESEEM (3P-ESEEM) spectra were collected using the pulse sequence $\pi/2-\tau-\pi/2$-$T-\pi/2-\tau$-echo where the delay time, T, was increased by 16 ns steps. 3P-ESEEM spectra were recorded at 10 K, $\tau = 128$ ns (values chosen to minimize proton modulations to the spectra), $\pi/2 = 12$ ns, and a microwave frequency of 9.816 GHz. Four-step phase cycling was used. Time-domain spectra were baseline-corrected with a second order exponential, apodized with a hamming window, zero-filled to 10-fold points, and fast Fourier-transformed (FFT) to yield the frequency-domain spectra. Spectral processing and simulations were performed using the EasySpin 4.0 toolbox in the Matlab R2017b software suite (The Mathworks Inc., Natick, MA).\textsuperscript{9}

**Analysis of H\textsubscript{2}\textsuperscript{34}S release by GC–MS.** To generate $^{34}$S-labeled L-cluster on $\text{MaNifB}$, 420 nmol reconstituted, DT-free $\text{MaNifB}$ was incubated with 10 mM SAM, 4 mM Eu\textsuperscript{II}-EGTA, 2 mM Na\textsubscript{2}$^{34}$SO\textsubscript{3} (Sigma-Aldrich), 50 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol in a total volume of 1 mL. In parallel, an unlabeled $\text{MaNifB}$ sample was prepared the same way as described above except for the substitution of 2 mM natural abundance Na\textsubscript{2}SO\textsubscript{3} for Na\textsubscript{2}$^{34}$SO\textsubscript{3}. Each reaction mixture was stirred for 10 min at room temperature under N\textsubscript{2} in an anaerobic chamber (Vacuum Atmosphere) with an O\textsubscript{2} level of <2 ppm, followed by purification of the $^{34}$S-labeled or unlabeled $\text{MaNifB}$ on a Ni-NTA column (GE Healthcare) in the absence of dithionite.

To release the cluster-bound sulfide as H\textsubscript{2}S, a total of 180 nmol of DT-free, $^{34}$S-labeled or unlabeled $\text{MaNifB}$ was treated with 30% trifluoracetic acid in a sealed 300-uL vial. The acid-treated sample was incubated at 60°C for 15 min to volatilize H\textsubscript{2}S before the entire headspace was injected by a gas-tight syringe into a GC–MS (Thermo-Fisher Scientific Trace 1300 GC connected to a Thermo-Fisher Scientific ISQ QD single quadrupole mass spectrometry) equipped with a Restek Rxi-1ms column (30 m, 0.32 mm ID, 4.0 $\mu$m df). The GC inlet and oven were maintained at a temperature of 30°C, and the mass spectrometry transfer line and ion source were maintained at a temperature of 250 °C. Total ion chromatograms were generated under SIM conditions in the EI mode, and H\textsubscript{2}$^{34}$S was detected at an m/z ratio of 36.

**Cluster Maturation Assays.** Cluster maturation assays were performed using a previously described protocol with slight modifications.\textsuperscript{8} The assay was initiated with treatment of 28
nmol reconstituted, DT-free *Ma*NifB with 5 mM SAM, 4 mM Eu\textsuperscript{II}-EGTA, 50 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol in a total volume of 300 µL at room temperature for 10 min. The samples containing SO\textsubscript{3}\textsuperscript{2−} and TeO\textsubscript{3}\textsuperscript{2−} were further incubated with 0.1, 0.5, 2 and 4 mM Na\textsubscript{2}SO\textsubscript{3} and Na\textsubscript{2}TeO\textsubscript{3}, respectively, at room temperature for 20 min. The samples containing SeO\textsubscript{3}\textsuperscript{2−} were incubated with 0.1, 0.25, 0.5, 1 and 2 mM Na\textsubscript{2}SeO\textsubscript{3} at room temperature for 20 min. Each incubation mixture was then combined with a 700-µL solution containing 6.7 nmol apo-NifEN, 16 nmol NifH, 1.4 nmol apo-NifDK, 2 mM Eu\textsuperscript{II}-EGTA, 0.5 mM homocitrate, 0.25 mM Na\textsubscript{2}MoO\textsubscript{4}, 0.8 mM Na\textsubscript{2}ATP, 1.7 mM MgCl\textsubscript{2}, 10 mM creatine phosphate, 8 units of creatine phosphokinase, and 50 mM Tris-HCl (pH 8.0). These reaction mixtures were incubated at 30°C for another 30 min before being examined for enzymatic activities as described previously. All activity assays were performed in the presence of 20 mM DT.

**Cluster Transfer Assays.** Each cluster transfer assay contained, in a total volume of 1 mL, 100 nmol non-tagged *Mt*NifB, 10 mM SAM, 4 mM Eu\textsuperscript{II}-EGTA, 50 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol. Following the initial incubation at room temperature for 15 min, the ‘+SO\textsubscript{3}\textsuperscript{2−}’, ‘+SeO\textsubscript{3}\textsuperscript{2−}’ and ‘+TeO\textsubscript{3}\textsuperscript{2−}’ assays were incubated with 2 mM Na\textsubscript{2}SO\textsubscript{3}, 0.5 mM Na\textsubscript{2}SeO\textsubscript{3} and 0.5 mM Na\textsubscript{2}TeO\textsubscript{3}, respectively, at room temperature for another 15 min; whereas the ‘−SO\textsubscript{3}\textsuperscript{2−}’ assay was not treated with any additive. Each assay was then mixed with 23 nmol DT-free, His-tagged apo-NifEN for 30 min to allow cluster transfer from NifB to NifEN, followed by loading of the mixture onto a Poly-Prep chromatography column (BioRad) packed with 1.4-mL Ni Sepharose 6 fast flow resin (GE Healthcare) to bind His-tagged NifEN. The column was washed with 5 mL buffer containing 0.5 mM Eu\textsuperscript{II}-EGTA, 10 mM imidazole, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10% (v/v) glycerol, and 5 mL buffer containing 2 mM dithionite, 10 mM imidazole, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10% (v/v) glycerol. The bound NifEN protein was then eluted from the column with a buffer containing 2 mM dithionite, 250 mM imidazole, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10% (v/v) glycerol. Each of the NifEN proteins reisolated from the ‘−SO\textsubscript{3}\textsuperscript{2−}’, ‘+SO\textsubscript{3}\textsuperscript{2−}’, ‘+SeO\textsubscript{3}\textsuperscript{2−}’ and ‘+TeO\textsubscript{3}\textsuperscript{2−}’ assays was subjected to cluster maturation and examined for its ability to donate M-clusters to apo-NifDK in an assay containing, in a total volume of 900 µL, 11 nmol NifEN, 28 nmol NifH, 2.1 nmol apo-NifDK, 20 mM dithionite, 0.5 mM homocitrate, 0.25 mM Na\textsubscript{2}MoO\textsubscript{4}, 0.8 mM Na\textsubscript{2}ATP, 1.7 mM MgCl\textsubscript{2}, 10 mM creatine phosphate, 8 units of creatine phosphokinase and 50 mM Tris-HCl (pH 8.0). The reaction mixture was incubated at 30°C for 30 min before it was examined for enzymatic activities as described previously.

**X-ray Absorption (XAS) Spectroscopy.** The XAS samples were prepared using a previously described protocol with slight modifications. The procedure began with incubation of 420
nmol reconstituted, DT-free *MaNifB* with 10 mM SAM, 4 mM Eu\(^{ll}\)-EGTA, 50 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol in a total volume of 1 mL at room temperature for 10 min. The sample containing SO\(_3^{2-}\) (\(MaNifB-L^S\)) was then incubated with 2 mM Na\(_2\)SO\(_3\), while the samples containing SeO\(_3^{2-}\) (\(MaNifB-L^Se\)) and TeO\(_3^{2-}\) (\(MaNifB-L^Te\)) were incubated with 0.5 mM Na\(_2\)SeO\(_3\) and Na\(_2\)TeO\(_3\), respectively, at room temperature for 20 min. Each incubation mixture was then concentrated to 180 µL and mixed with a 30-µL buffer containing 50 mM Tris-HCl (pH 8.0) and 80% (v/v) glycerol. The resulting mixture was transferred into an XAS sample cell, frozen in liquid nitrogen, and stored in a liquid nitrogen dewar until use.

Fe, Se, and Te K-edge X-ray absorption spectra were collected either on SSRL beam line 9-3 using a 100-element Ge monolith solid-state detector (Canberra) or beam line 7-3 using a 30-element solid-state Ge detector (Canberra) with a SPEAR3 storage ring current of ~500 mA at an energy of 3.0 GeV. The BL9-3 optics consists of a flat, bent, harmonic rejection vertically collimating Rh-coated Si \(M_0\) mirror, a liquid nitrogen cooled double crystal Si(220), monochromator, fully tuned, and a post-monochromator, bent, cylindrical, Rh-coated Si focusing \(M_1\) mirror. The BL7-3 optics consists of a flat, bent, harmonic rejection vertically collimating Rh-coated Si \(M_0\) mirror, and a liquid nitrogen cooled double crystal Si(220) monochromator. All Fe K-edge scans were taken between 6882 and 8000 eV, all Se K-edge scans were taken between 12420 and 13540 eV, and all Te K-edge scans were taken between 31570 and 32700 eV at ~10 K using an Oxford Instruments CF1208 continuous flow liquid helium cryostat using an open-cycle liquid He dewar (BL 9-3) or cooled by a closed-cycle cooled He gas loop (BL 7-3). For Fe scans, an iron foil was placed in the beam pathway prior to the ionization chamber \(I_0\) and scanned concomitantly for an energy calibration, with the first inflection point of the edge assigned to 7112.0 eV. For Se scans, a Se standard was placed between the ionization chambers \(I_1\) and \(I_2\) and scanned concomitantly for an energy calibration, with the first inflection point of the edge assigned to 12658.0 eV. For Te scans, a Te standard (TeO\(_2\) diluted in boron nitride) was placed between the ionization chambers \(I_1\) and \(I_2\) and scanned concomitantly for an energy calibration, with the center of the edge assigned to 31818.0 eV. A Soller slit with a Z-1 filter was used to increase the signal to noise ratio of the spectra. Photoreduction was monitored by scanning the same spot on the sample twice and comparing the first derivative peaks associated with the edge energy during data collection. The scan information is described in Supplementary Table 7 below.

The detector channels from the scans were examined, calibrated and averaged using EXAFSPAK,\(^{11}\) then processed for EXAFS analysis using PYSPLINE\(^{12}\) to extract \(\chi(k)\). PYSPLINE was used to subtract a second-order background from the entire range of data, then
generate a spline function to model background absorption through the EXAFS region. A four-
region spline was chosen with 2, 3, 3 order polynomials over the post edge region, and the data
were normalized to have an edge jump of 1.0 at 7130 eV for Fe, 12675 eV for Se and 31830
eV for Te. Following a previously reported data analysis protocol, the Fe K-edge EXAFS
data for the MaNifB-bound LSe-cluster (MaNifB-LSe) and MaNifB-bound LTE-cluster
(MaNifB-LTe) were generated by subtracting the k-weighted EXAFS data, \( \chi(k) \), of the MaNifB
variant (containing the SAM-cluster but no K-cluster) from the \( \chi(k) \) of the wildtype MaNifB
(containing both SAM- and K-clusters) incubated with SAM and SeO\(_3^{2-}\) and TeO\(_3^{2-}\),
respectively, in a 1:2 ratio, based on the proportionate iron quantity for each cluster species
(\textit{i.e.}, 4 Fe for the SAM-cluster and 8 Fe for the LSe- and LTe-clusters). Theoretical phase
and amplitude parameters for a given absorber-scatterer pair were calculated using FEFF 8.40
and subsequently applied to the nonlinear least squares ‘opt’ fitting program of the EXAFSPAK
package during curve fitting. Parameters for each species were calculated using an appropriate
model derived from either the crystal structure of the M-cluster in NifDK (PDB code 3U7Q),
where the Mo atom was exchanged for an Fe and a Se/Te atom was added in the belt sulfur
position since high-resolution crystal structures of MaNifB are not yet available. In all analyses,
the coordination number of a given shell (N) was a fixed parameter and was varied iteratively
in integer steps, whereas the bond length (R) and mean-square deviation (\( \sigma^2 \)) were allowed to
freely float. The estimated uncertainties of R, \( \sigma^2 \), and N are 0.02 Å, 0.1 \( \times 10^{-3} \) Å\(^2\), and 20%,
respectively. The amplitude reduction factor \( S_0 \) was fixed at 1.0 for all K-edge data, whereas
the edge-shift parameter \( \Delta E_0 \) was allowed to float as a single value for all shells. Thus, in any
given fit, the number of floating parameters was typically equal to \( 2 \times \) number of shells + 1.
The goodness of fit (GOF) parameters were calculated as follows:

\[
F = \sqrt{\sum k^6 (\chi_{\text{exp}} - \chi_{\text{calc}})^2}
\]

\[
F' = \sqrt{\sum k^6 \left( \chi_{\text{exp}} - \chi_{\text{calc}} \right)^2 / \sum k^6 \chi_{\text{exp}}^2}
\]

Since the MaNifB samples incubated with SeO\(_3^{2-}\) and TeO\(_3^{2-}\) contained Eu\(^{II}\)-EGTA, the Eu L-
edge signal interfered with the Fe K-edge data collected above \~7630 eV. Consequently, the Fe
K-edge data were truncated at 7605 eV and analyzed with a \( k \) range of 2–11.2 Å\(^{-1}\) (\( \Delta R = 0.17 \)
Å) to permit comparison between all cluster species. The Se and Te K-edge data were
unaffected by the presence of Eu\(^{II}\)-EGTA.
Density functional theory calculations. Density functional theory (DFT) calculations were carried out with the DFT programs in the Turbomole package, version 7.0.$^{15}$ An atomistic model of the L-cluster (including a central carbide atom) was built with Molden.$^{16}$ For calculations of the energetics of XO$_3^{2-}$ coordination (X: S, Se, Te), one S$^{2-}$ of the cluster was replaced by either compound in separate calculations. For the intact L-cluster, an overall charge of -4 elementary charges [Fe(II)$_6$Fe(III)$_2$S$_9$C] was assumed. The EPR data suggest the cluster to be in a singlet spin state. The antiferromagnetic coupling resulting in this state was accounted for by the broken symmetry approach.$^{17-19}$ Solvent effects were calculated implicitly by the conductor-like solvent screening model (COSMO)$^{20}$ as implemented in Turbomole, with a dielectric constant of $\varepsilon = 20$. The models were treated as open-shell systems in the unrestricted Kohn-Sham framework. Structural optimizations were performed with the TPSS functional$^{21}$ and a def2-TZVP$^{22,23}$ basis set assigned to all model atoms. Computational time was reduced by utilizing the resolution-of-the-identity approximation.$^{24,25}$ Dispersion interactions were accounted for by the dispersion correction DFT-D3$^{26}$ as implemented in Turbomole. For the reduction steps leading from the initially coordinated XO$_3^{2-}$ species to the final X$^{2-}$ forms, coupled e$^-$/H$^+$ transfer was assumed, thereby leaving the charge of the cluster unchanged throughout the process and reflecting the excess of reductant present in the experimental setup. To calculate reaction energies, the electronic energies of reactants and products were considered, as well as the deprotonation energy of TrisH$^+$ obtained from separate DFT calculations. In order to obtain the approximate redox free energies that better describe the energetics of the system, the resulting energies were then corrected with the reported experimental electrode potential of Eu$^{II}$-DTPA ($E_{SHE}^0 = -1.14$ V vs. SHE).$^{27,28}$ This value was adapted to the buffer system with a previously reported value$^{29}$ for the relative electrode potential, $\Delta E_{SHE} = -4.34$ V. The initially obtained reduction energies were transformed into redox free energies by adding the redox free energy of the reductant half reaction: $\Delta G_R = -z F (E_{SHE}^0 - \Delta E_{SHE})$, where z is the number of electrons: 2 (transfer of two electrons/protons to dissociate one O atom as H$_2$O) and F is Faraday’s constant.$^{27,28}$ For Eu$^{II}$-DTPA, this value is -147.7 kcal/mol.
Supplementary Fig. 1. Maturation of the M-cluster of nitrogenase. (a) Pathway of M-cluster biosynthesis and (b) proposed mechanism of radical SAM-dependent carbide insertion during the process of L-cluster formation on NifB. (a) Biosynthesis of the M-cluster is initiated with the concerted actions of NifU/S, which sequentially generate [Fe₂S₂] and [Fe₄S₄] clusters. Subsequently, a pair of [Fe₄S₄] clusters (K-cluster) are transferred to NifB, where they are coupled and rearranged into an [Fe₈S₈C] precursor (L*-cluster) concomitant with insertion of an interstitial carbide. This step is followed by insertion of a ‘9th sulfur’ into the L*-cluster that gives rise to an [Fe₈S₉C] precursor (L-cluster) on NifB, and transfer of the L-cluster to NifEN, where the L-cluster is matured into a fully assembled, [(R-homocitrate)MoFe₇S₉C] cofactor (M-cluster) via NifH-mediated insertion of Mo and homocitrate (hc). Finally, the M-cluster is transferred from NifEN to its target location in NifDK, thereby completing the assembly process of the nitrogenase cofactor. (b) NifB contains three [Fe₄S₄] clusters: the RS-cluster (or SAM-cluster) and the two 4Fe modules of the K-cluster, designated the K1- and K2-clusters, respectively. Cleavage of the first SAM equivalent results in the formation of SAH and the transfer of a methyl group to a sulfide atom of K2 via an S₈₂-type mechanism. Cleavage of the second SAM equivalent results in the formation of an RS-cluster-bound methionine (Met) and a 5'-deoxyadenosyl radical (5'-dA•) that abstracts a hydrogen atom from the K2-bound methyl group, generating a K2-bound methylene radical. Continued deprotonation of the K2-bound methylene radical, facilitated by the His ligand of K1, which eventually gives rise to an interstitial carbide concomitant with the coupling and rearrangement of K1 and K2 into an [Fe₈S₈C] L*-cluster and Insertion of a ‘9th sulfur’ that converts the L*-cluster into an [Fe₈S₉C] L-cluster.
Supplementary Fig. 2. Speciation- and concentration-dependent incorporation of S, Se and Te into the L*-cluster. (a) Maturation activity of the MaNifB-bound K-cluster into an M-cluster upon incubation with SAM alone, SAM plus SO$_3^{2-}$, SAM plus SeO$_3^{2-}$, SAM plus TeO$_3^{2-}$, SAM plus Se$_2^{-}$ and SAM plus Te$_2^{-}$. Eu$^{II}$-EGTA, a sulfur-free reductant, was used in these assays. (b) Titration of the maturation activity of SAM-treated MaNifB against increasing concentrations of SO$_3^{2-}$, SeO$_3^{2-}$ or TeO$_3^{2-}$ in the presence of Eu$^{II}$-EGTA. Activities were normalized based on the L-cluster contents. (c) GC-MS analysis showing the H$_2$S standard (1) and the release of H$_2$S from MaNifB treated with SAM plus $^{32}$SO$_3^{2-}$ (2) or $^{34}$SO$_3^{2-}$ (3) in the presence of Eu$^{II}$-EGTA, followed by acid quenching. H$_2^{34}$S was traced at $m/z = 36$. Activity data (a, b) were obtained from three independent experiments (n=6) and presented as mean±s.d. The H$_2$S release experiments (c) were repeated three times (n=3), and representative GC-MS traces are presented.
Supplementary Fig. 3. Proposed model of stepwise N\textsubscript{2} reduction via cluster rotation. (a) Side and (b) top views of the M-cluster, showing rotation of the cluster in the direction of S3A→ S2B→ S5A that permits binding of N\textsubscript{2} at S3A (Â), reduction of N\textsubscript{2} to N\textsubscript{2}* (N\textsubscript{2}H\textsubscript{2}) at S2B (C), and further reduction of N\textsubscript{2}* (N\textsubscript{2}H\textsubscript{2}) to NH\textsubscript{3} at S5A (E). The final reduction steps at S5A signals loading of the ‘next’ N\textsubscript{2} at S3A via sulfur displacement (F), and subsequent release of NH\textsubscript{3} necessitates a ‘refill’ of sulfur at S5A (N) that allows continued cluster rotation. Rotation of each M-cluster is enabled by an alternating elongation/breakage of the Mo-O\textsubscript{7} (‘facing’ S2B) and Mo-O\textsubscript{5} (‘facing’ S5A) bonds, leading to a repetition of the cluster conformation between the S2B- (A-state) and S3A/S5A- (C-state) displaced states. Asynchronous rotation of the two M-clusters (M\textsuperscript{A} and M\textsuperscript{C}) in the two dimers of NifDK also occurs via alternating elongation/breakage of the Mo-O\textsubscript{7} and Mo-O\textsubscript{5} bonds in the two M-clusters, leading to an alternation of M\textsuperscript{A} and M\textsuperscript{C} between the A- and C-states as that captured in the N\textsubscript{2}-bound NifDK structure under limited turnover conditions.\textsuperscript{30}
Supplementary Tables

**Supplementary Table 1.** Best fits of the Fe K-edge EXAFS data ($k = 2$–$11.2$ Å$^{-1}$) of various *Ma*NifB-bound precursor species of the M-cluster.

| Species        | Fe–S |   | Fe•••Fe |   | Fe••••Fe |   |
|----------------|------|---|---------|---|----------|---|
|                | N    | R(Å) | $\sigma^2 (10^{-3})$ | N | R(Å) | $\sigma^2 (10^{-3})$ | N | R(Å) | $\sigma^2 (10^{-3})$ |
| *Ma*NifB-K [$^a$] | 3.8  | 2.29 | 8.19 | 1 | 2.51 | 5.82 | 1.5 | 2.69 | 4.35 |
| *Ma*NifB-L* [$^a$] | 3    | 2.24 | 4.95 | 3 | 2.62 | 9.30 | 1.2 | 3.69 | 6.96 |
| *Ma*NifB-L$_S$ [$^a$] | 3.1  | 2.23 | 4.23 | 3.5 | 2.64 | 7.87 | 1.5 | 3.70 | 7.89 |
| *Ma*NifB-L$_{Se}$ [$^b$] | 3    | 2.23 | 2.92 | 3.5 | 2.64 | 8.99 | 1.2 | 3.71 | 1.29 |
|                 |      | 0.8 | 3.89 | 1.12 |
| *Ma*NifB-L$_{Te}$ [$^c$] | 2    | 2.23 | 1.62 | 3.5 | 2.62 | 9.80 | 1 | 3.70 | 8.55 |

[$^a$]Taken from reference 10
[$^b$]Best fits from Supplementary Table 2
[$^c$]Best fits from Supplementary Table 3
**Supplementary Table 2.** Fit parameters for the Fe K-edge EXAFS data of MaNifB-L\textsuperscript{Se} between \(k = 2\)–\(11.2\) Å\(^{-1}\) (\(E_0 = 7130.0\) eV). The fit parameters R, \(\sigma^2\), and N have estimated uncertainties of \(\pm0.02\) Å, \(\pm0.1 \times 10^{-3}\) Å\(^2\), and 20%, respectively. Fit 16 gives the best fit of the experimental data.

|    | Fe-S | Fe\(\cdot\cdot\cdot\)Fe | Fe\(\cdot\cdot\cdot\)Fe | GOF |
|----|------|----------------|----------------|-----|
|    | N    | R(Å) | \(\sigma^2\)\((10^{-3})\) | N    | R(Å) | \(\sigma^2\)\((10^{-3})\) | N    | R(Å) | \(\sigma^2\)\((10^{-3})\) | Δ\(E_0\) | F    | F' \((\times10^3)\) |
| 1  | 2    | 2.21 | 1.06 |       |       |       |       |       |       | 15.9  | 876  | 556  |
| 2  | 3    | 2.21 | 3.81 |       |       |       |       |       |       | 14.6  | 867  | 553  |
| 3  | 4    | 2.22 | 6.15 |       |       |       |       |       |       | 13.5  | 921  | 570  |
| 4  | 4    | 2.25 | 5.86 | 1.266 | 0.47 |       |       |       |       | -7.84 | 275  | 312  |
| 5  | 4    | 2.24 | 5.47 | 2.266 | 4.71 |       |       |       |       | -7.92 | 288  | 319  |
| 6  | 3    | 2.24 | 3.20 | 2.265 | 5.08 |       |       |       |       | -8.45 | 208  | 271  |
| 7  | 3    | 2.23 | 3.17 | 3.265 | 8.04 |       |       |       |       | -8.40 | 225  | 282  |
| 8  | 3    | 2.23 | 3.14 | 3.8264 | 10.11 |       |       |       |       | -8.69 | 253  | 299  |
| 9  | 3    | 2.23 | 3.06 | 3.8264 | 9.96 | 3.71 | 4.79 |       |       | -8.68 | 225  | 281  |
| 10 | 3    | 2.23 | 3.09 | 3.8264 | 9.95 | 3.72 | 8.33 |       |       | -8.61 | 231  | 285  |
| 11 | 3    | 2.23 | 2.94 | 3.8264 | 9.72 | 0.83 | 1.29 | -0.80 | -8.50 | 209  | 271  |
|    |      |      |      |       |       |       |       |       |       |       | 211  | 273  |
| 12 | 3    | 2.23 | 2.95 | 3.8264 | 9.75 | 1.23 | 1.89 | -0.80 | -8.65 |       |       | 211  | 273  |
| 13 | 3    | 2.23 | 3.18 | 2.98 | 3.74 | 3.90 | 1.00 |       |       |       |       |       | 158  | 236  |
| 14 | 3    | 2.23 | 3.37 | 3.00 | 2.46 | 3.72 | 8.88 | -9.65 | 169   | 244  |       |       |       |       |
| 15 | 3    | 2.23 | 3.33 | 3.5 | 2.65 | 9.27 | 3.72 | 8.87 | -8.41 | 231  | 285  |       |       |       |
| 16 | 3    | 2.23 | 2.92 | 3.5 | 2.64 | 8.99 | 1.23 | 1.29 | -8.47 | 198  | 264  |       |       |       |
| 17 | 3    | 2.23 | 3.08 | 3.5 | 2.65 | 8.84 | 1.23 | 1.23 | -8.26 | 212  | 273  |       |       |       |
Supplementary Table 3. Fit parameters for the Fe K-edge EXAFS data of MaNifB-LTe between $k = 2$-11.2 Å$^{-1}$ ($E_0 = 7130.0$ eV). The fit parameters $R$, $\sigma^2$, and $N$ have estimated uncertainties of ±0.02 Å, ±0.1 × 10$^{-3}$ Å$^2$, and 20%, respectively. Fit 18 gives the best fit of the experimental data.

|   | Fe-S | Fe•••Fe |   | Fe•••Fe | GOF |
|---|------|---------|---|---------|-----|
|   | N    | R(Å)    | $\sigma^2$(10$^{-3}$) | N | R(Å)    | $\sigma^2$(10$^{-3}$) | N | R(Å)    | $\sigma^2$(10$^{-3}$) | $\Delta E_0$ | F | F' (x10$^3$) |
| 1 | 2    | 2.22    | 2.76                       |   |   |   |   |   |   |   |   |   |   |
| 2 | 3    | 2.23    | 5.85                       |   |   |   |   |   |   |   |   |   |   |
| 3 | 4    | 2.24    | 8.56                       |   |   |   |   |   |   |   |   |   |   |
| 4 | 3    | 2.26    | 4.76                       | 1 | 2.65 | 0.87 |   |   |   |   |   |   |   |
| 5 | 3    | 2.25    | 4.52                       | 2 | 2.65 | 5.27 |   |   |   |   |   |   |   |
| 6 | 3    | 2.24    | 4.58                       | 3 | 2.64 | 8.41 |   |   |   |   |   |   |   |
| 7 | 3    | 2.24    | 4.76                       | 3.8| 2.64 | 10.59|   |   |   |   |   |   |   |
| 8 | 3    | 2.24    | 4.70                       | 3.8| 2.64 | 10.61| 1 | 3.72 | 10.77| -8.71 | 225 | 326 |
| 9 | 3    | 2.25    | 4.87                       | 3.3| 2.66 | 9.53 | 1 | 2.93 | 7.11  | -8.43 | 156 | 271 |
|   | 0.5  | 3.03    | 9.80                       |   |   |   |   |   |   |   |   |   |   |
| 10| 3    | 2.24    | 4.55                       | 3 | 2.64 | 8.40 | 0.8| 3.69 | 3.86  | -8.36 | 183 | 295 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 11| 3    | 2.24    | 4.69                       | 3.8| 2.64 | 10.45| 0.8| 3.70 | 4.49  | -8.27 | 224 | 325 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 12| 3    | 2.24    | 4.62                       | 3.5| 2.64 | 9.68 | 0.8| 3.71 | 4.40  | -8.27 | 208 | 314 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 13| 3    | 2.24    | 4.81                       | 3 | 2.65 | 9.19 | 0.8| 3.72 | 3.13  | -8.37 | 149 | 266 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 14| 3    | 2.24    | 4.89                       | 3 | 2.65 | 9.40 | 0.8| 3.72 | 5.78  | -8.44 | 148 | 265 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 15| 2    | 2.23    | 1.67                       | 3.8| 2.63 | 10.49| 1 | 3.71 | 9.08  | -9.58 | 132 | 249 |
| 16| 2    | 2.23    | 1.55                       | 3.8| 2.63 | 10.30| 0.8| 3.69 | 1.96  | -9.54 | 124 | 242 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 17| 2    | 2.23    | 1.57                       | 3 | 2.62 | 8.83 | 0.8| 3.69 | 1.52  | -10.1 | 99  | 216 |
|   |      |         |                            |   |   |   |   |   |   |   |   |   |   |
| 18| 2    | 2.23    | 1.62                       | 3.5| 2.62 | 9.80 | 1 | 3.70 | 8.55  | -9.71 | 125 | 243 |
**Supplementary Table 4.** Best fits of the Se and Te K-edge EXAFS data (k = 4–15 Å⁻¹) of various MaNifB-bound precursor species of the M-cluster.

| Species         | Se-Fe | Te-Fe |
|-----------------|-------|-------|
|                 | N     | R(Å)  | σ² (10⁻³) | N     | R(Å)  | σ² (10⁻³) |
| MaNifB-L⁵⁷Se    | 2     | 2.38  | 3.91     | N/A   |       |          |
| MaNifB-L⁵⁷Te    |       | N/A   |          | 1     | 2.55  | 5.45     |
|                 |       |       |          | 2     | 2.79  | 1.61     |

*Best fits from Supplementary Table 5

*Best fits from Supplementary Table 6*

**Supplementary Table 5.** Fit parameters for the Se K-edge EXAFS data of MaNifB-L⁵⁷Se between k = 4-15 Å⁻¹ (E₀ = 12675.0 eV). The fit parameters R, σ², and N have estimated uncertainties of ±0.02 Å, ±0.1 × 10⁻³ Å², and 20%, respectively. Fit 2 gives the best fit of the experimental data.

| Fit | N | R(Å) | σ² (10⁻³) | N | R(Å) | σ² (10⁻³) | ΔE₀ | F (x10^3) | F' (x10^3) |
|-----|---|------|-----------|---|------|-----------|-----|-----------|------------|
| 1   | 1 | 2.38 | 1.26      |   |      |           | -12.1| 273       | 369        |
| 2   | 2 | 2.38 | 3.91      |   |      |           | -10.9| 163       | 285        |
| 3   | 3 | 2.39 | 6.12      |   |      |           | -9.34| 298       | 385        |
| 4   | 2 | 2.38 | 3.92      | 1 | 2.07 | 9.59      | -10.8| 147       | 270        |
| 5   | 2 | 2.38 | 4.04      | 0.5| 2.07 | 2.21      | -9.94| 145       | 269        |
Supplementary Table 6. Fit parameters for the Te K-edge EXAFS data of MaNifB-LTe between $k = 4-15\ \text{Å}^{-1}$ ($E_0 = 31830.0\ \text{eV}$). The fit parameters $R$, $\sigma^2$, and $N$ have estimated uncertainties of ±0.02Å, ±0.1 × $10^{-3}$Å$^2$, and 20%, respectively. Fit 5 gives the best fit of the experimental data.

| Fit | N | R(Å) | $\sigma^2$(10$^{-3}$) | N | R(Å) | $\sigma^2$(10$^{-3}$) | $\Delta E_0$ (x10$^3$) | F | F' |
|-----|---|------|------------------------|---|------|------------------------|------------------------|---|---|
| 1   | 1 | 2.78 | -0.90                 | 1 | 2.52 | 12.29                 | 11.9                   | 454| 503|
| 2   | 2 | 2.80 | 1.46                  | 1 | 2.56 | 11.32                 | 16.7                   | 524| 539|
| 3   | 3 | 2.81 | 3.37                  | 2 | 2.57 | 23.01                 | 21.1                   | 759| 650|
| 4   | 1 | 2.79 | -0.80                 | 1 | 2.52 | 12.9                  | 16.7                   | 437| 492|
| 5   | 2 | 2.79 | 1.61                  | 1 | 2.55 | 5.45                  | 11.9                   | 454| 503|
| 6   | 2 | 2.78 | 1.28                  | 2 | 2.56 | 11.32                 | 16.7                   | 524| 539|
| 7   | 1 | 2.78 | -0.90                 | 2 | 2.57 | 23.01                 | 21.1                   | 759| 650|
| 8   | 2 | 2.79 | 1.67                  | 1 | 2.08 | 31.96                 | 16.5                   | 389| 465|
| 9   | 2 | 2.79 | 1.73                  | 0.5| 2.08 | 3.84                  | 17.5                   | 392| 467|
| 10  | 2 | 2.79 | 1.75                  | 0.5| 2.06 | 0.48                  | 16.4                   | 381| 460|
| 11  | 2 | 2.79 | 1.74                  | 0.5| 2.08 | 1.69                  | 17.01                  | 389| 465|

Supplementary Table 7. Summary of XAS scan information for MaNifB samples.

| Sample                  | Beam Line | Edge | Scans | Filter     |
|-------------------------|-----------|------|-------|------------|
| MaNifB + SAM + SeO$_3^{2-}$ | 9-3       | Fe   | 8     | Mn 3 µm    |
| MaNifB + SAM + SeO$_3^{2-}$ | 9-3       | Se   | 8     | As 3 µm    |
| Na$_2$SeO$_3$ in buffer | 9-3       | Se   | 6     | As 3 µm    |
| MaNifB + SAM + TeO$_3^{2-}$ | 9-3       | Fe   | 8     | Mn 3 µm    |
| MaNifB + SAM + TeO$_3^{2-}$ | 7-3       | Te   | 8     | Sb 3 µm    |
| Na$_2$TeO$_3$ in buffer | 7-3       | Te   | 6     | Sb 3 µm    |
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