Probing the coordination and function of Fe$_4$S$_4$ modules in nitrogenase assembly protein NifB

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NifB is an essential radical S-adenosylmethionine (SAM) enzyme for nitrogenase cofactor assembly. Previous studies show that NifB couples a putative pair of [Fe$_4$S$_4$] modules (designated K1 and K2) into an [Fe$_8$S$_9$C] cofactor precursor concomitant with radical SAM-dependent carbide insertion through the action of its SAM-binding [Fe$_4$S$_4$] module. However, the coordination and function of the NifB cluster modules remain unknown. Here, we use continuous wave and pulse electron paramagnetic resonance spectroscopy to show that K1- and K2-modules are 3-cysteine-coordinated [Fe$_4$S$_4$] clusters, with a histidine-derived nitrogen serving as the fourth ligand to K1 that is lost upon K1/K2-coupling. Further, we demonstrate that coexistence of SAM/K2-modules is a prerequisite for methyltransfer to K2 and hydrogen abstraction from the K2-associated methyl by a 5′-deoxyadenosyl radical. These results establish an important framework for mechanistic explorations of NifB while highlighting the utility of a synthetic-cluster-based reconstitution approach employed herein in functional analyses of iron–sulfur (FeS) enzymes.
Nitrorgenase is a complex metalloenzyme that catalyzes the remarkable chemical transformations of N₂ to NH₃, and CO to hydrocarbons, at its cofactor site. Designated the M-cluster, the cofactor of the Mo-nitrogenase is a [MoFe₇S₉C(+R-homocitrate)] cluster comprising [MoFe₅S₆] and [Fe₄S₄] sub-clusters ligated by three μ₃–sulfides and one μ₃–interstitial carbide, with an R-homocitrate moiety providing further coordination to its terminal Mo atom. The unique reactivity of M-cluster has sparked interest in elucidating its biosynthetic mechanism. Such knowledge is crucial for understanding the structure–function relationship of nitrogenase, which in turn could facilitate future development of biomimetic catalysts for ambient conversion of N₂ and CO substrates into valuable chemical commodities. Previous studies have led to the proposal of a working model of M-cluster assembly (Supplementary Fig. 1)⁹–⁰⁰. The early stage of this process involves the coupling and rearrangement of two putative [Fe₄S₄] clusters (designated the K-cluster) on an assembly scaffold. NiFB, concomitant with the insertion of an interstitial carbide and a 9th sulfur, giving rise to a [Fe₅S₅C] core (designated the L-cluster) that is nearly indistinguishable in structure from the M-cluster (Supplementary Fig. 1a, i). Subsequently, the 8Fe L-cluster is matured into an M-cluster on another assembly scaffold, NiFEN, upon substitution of a Mo-homocitrate conjugate for one terminal Fe atom of the cluster by NiFHT, the cofactor maturation (Supplementary Fig. 1a, iv). Finally, the M-cluster is transferred to its target binding site in NiDK, the catalytic component of Mo-nitrogenase (Supplementary Fig. 1a, v).

Of all components along the M-cluster assembly pathway, NiFB plays a pivotal role in transforming small, putative Fe₄S₄ units (K-cluster) into an 8Fe entity (L-cluster) that has the complete FeS core structure in place, as well as the interstitial carbide. A member of the S-adenosyl-l-methionine (SAM) enzyme family. NiFB carries the signature CxxxCxxxC radical SAM motif, as well as a number of ligands that could accommodate coordination of the entire complement of Fe atoms of the M-cluster. Characterization of the NiFB proteins from Azoto bacter vinelandii¹¹–¹³ and Methanosarcina acetivorans¹⁴ has unveiled a radical SAM-dependent mechanism employed by NiFB for carbide insertion, which begins with methyltransfer in an SN₂-type mechanism from SAM to a putative [Fe₄S₄] cluster pair (Supplementary Fig. 1b, i). Subsequently, the transferred methyl group undergoes hydrogen abstraction by a SAM-derived 5′-deoxyadenosyl [5′-dA•+] radical (Supplementary Fig. 1b, ii). This step is followed by further deprotonation and/or dehydrogenation of the resultant carbon radical, which occurs concomitantly with the coupling and rearrangement of the putative[Fe₄S₄] clusters pair into an [Fe₅S₅C] core via radical chemistry (Supplementary Fig. 1b, iii). Together, these results suggest the presence and concerted action of three 4Fe modules, namely, the two 4Fe modules (designated the K1- and K2-modules) that give rise to the K-cluster and a third 4Fe module that is ligated by the radical SAM motif (designated the SAM-module), on NiFB. However, despite the past and recent progress toward elucidating the NiFB-catalyzed reaction¹¹–¹⁴, questions remain open as to the 4Fe nature of the NiFB-associate clusters. A series of variants—each carrying one of the three proposed 4Fe modules—was generated on the template of the NiFB protein from M. acetivorans (designated MaNiFB). Previously, MaNiFB was successfully expressed in Escherichia coli¹⁴. Sequence analysis of MaNiFB reveals the presence of three groups of highly conserved Cys residues—three Cys residues per group—that could potentially serve as ligands for the SAM- (K1- and K2-modules of NiFB (Fig. 1a, b)). Specifically, Cys¹⁵,²⁰, Cys³⁰, and Cys³⁷, which form the canonical CxxxCxxxC radical SAM motif, are assigned to the SAM-module; Cys⁴⁰, Cys⁶³, and Cys³²⁹, which are located toward the N terminus of the primary sequence, are assigned to the K1 module; and Cys²⁶⁴, Cys²⁷⁴, and Cys²⁷⁷, which are positioned toward the C terminus of the primary sequence, are assigned to the K2 module (Fig. 1a, b). On the basis of the tentative assignment of the Cys residues, each cluster-binding module of MaNiFB can be studied independently by mutating the Cys ligands of the other two modules to Ala. Using this approach, we generated three constructs, each encoding a MaNiFB variant with a single SAM (designated MaNiFB²SAM), K1 (designated MaNiFB²K1), or K2 (designated MaNiFB²K2) module (see Supplementary Fig. 2, i–iii). We then co-expressed each MaNiFB variant with the FeS-assembly machinery, IscSU, in E. coli. Purified MaNiFB variants, as their native counterpart, showed a monomeric composition comprising a 38 kDa subunit (Supplementary Fig. 3). However, the cluster contents of the as-purified MaNiFB proteins were insufficient for spectroscopic and biochemical analyses. To circumvent this problem, we used a recently developed protocol to first remove the endogenous Fe₅S₆ clusters of the MaNiFB variants and then reconstitute these proteins with a synthetic [Fe₄S₄] compound (see Supplementary Fig. 2). Such a reconstitution approach has been successfully applied to identify the source of the 9th sulfur during the cofactor maturation process on MaNiFB without interference of the Fe/S impurities often introduced by the traditional FeCl₃/Na₂S-based reconstitution method and, in this particular case, it can be used to conclusively determine the [Fe₄S₄] identity of the individual modules in MaNiFB. Indeed, upon in vitro reconstitution with this synthetic [Fe₄S₄] compound, each of the three MaNiFB variants has an S = 1/2 electron paramagnetic resonance (EPR) signal that is characteristic of a [Fe₄S₄]⁺ cluster when the protein is reduced by dithionite (Fig. 1c; also see Supplementary Table 1 for Fe contents, spin concentrations and protein concentrations of EPR samples). Consistent with their different origins, the simulated spectra have distinct g values from one another (MaNiFB²SAM: g = [2.017 1.924 1.910]; MaNiFB²K1: g = [2.050 1.905 1.900]; MaNiFB²K2: g = [2.044 1.933 1.886]; also see Supplementary Fig. 4 and Supplementary Table 2 for simulation parameters), and the contributions of most EPR features of the individual modules can be identified in the spectrum of the wild-type MaNiFB (Fig. 1c). These observations are exciting, as they provide strong support for the assignment of the respective Cys residues as the ligands of the three modules in MaNiFB while supplying direct proof for the [Fe₄S₄] identity of the K1- and K2-modules.

Results

Establishing the 4Fe nature of the NiFB-associated clusters.

A series of variants—each carrying one of the three proposed 4Fe modules—was generated on the template of the NiFB protein from M. acetivorans (designated MaNiFB). Previously, MaNiFB was successfully expressed in Escherichia coli¹⁴. Sequence analysis of MaNiFB reveals the presence of three groups of highly conserved Cys residues—three Cys residues per group—that could potentially serve as ligands for the SAM- (K1- and K2-modules of NiFB (Fig. 1a, b)). Specifically, Cys¹⁵,²⁰, Cys³⁰, and Cys³⁷, which form the canonical CxxxCxxxC radical SAM motif, are assigned to the SAM-module; Cys⁴⁰, Cys⁶³, and Cys³²⁹, which are located toward the N terminus of the primary sequence, are assigned to the K1 module; and Cys²⁶⁴, Cys²⁷⁴, and Cys²⁷⁷, which are positioned toward the C terminus of the primary sequence, are assigned to the K2 module (Fig. 1a, b). On the basis of the tentative assignment of the Cys residues, each cluster-binding module of MaNiFB can be studied independently by mutating the Cys ligands of the other two modules to Ala. Using this approach, we generated three constructs, each encoding a MaNiFB variant with a single SAM (designated MaNiFB²SAM), K1 (designated MaNiFB²K1), or K2 (designated MaNiFB²K2) module (see Supplementary Fig. 2, i–iii). We then co-expressed each MaNiFB variant with the FeS-assembly machinery, IscSU, in E. coli. Purified MaNiFB variants, like their native counterpart, showed a monomeric composition comprising a 38 kDa subunit (Supplementary Fig. 3). However, the cluster contents of the as-purified MaNiFB proteins were insufficient for spectroscopic and biochemical analyses. To circumvent this problem, we used a recently developed protocol to first remove the endogenous Fe₅S₆ clusters of the MaNiFB variants and then reconstitute these proteins with a synthetic [Fe₄S₄] compound (see Supplementary Fig. 2). Such a reconstitution approach has been successfully applied to identify the source of the 9th sulfur during the cofactor maturation process on MaNiFB without interference of the Fe/S impurities often introduced by the traditional FeCl₃/Na₂S-based reconstitution method and, in this particular case, it can be used to conclusively determine the [Fe₄S₄] identity of the individual modules in MaNiFB. Indeed, upon in vitro reconstitution with this synthetic [Fe₄S₄] compound, each of the three MaNiFB variants has an S = 1/2 electron paramagnetic resonance (EPR) signal that is characteristic of a [Fe₄S₄]⁺ cluster when the protein is reduced by dithionite (Fig. 1c; also see Supplementary Table 1 for Fe contents, spin concentrations and protein concentrations of EPR samples). Consistent with their different origins, the simulated spectra have distinct g values from one another (MaNiFB²SAM: g = [2.017 1.924 1.910]; MaNiFB²K1: g = [2.050 1.905 1.900]; MaNiFB²K2: g = [2.044 1.933 1.886]; also see Supplementary Fig. 4 and Supplementary Table 2 for simulation parameters), and the contributions of most EPR features of the individual modules can be identified in the spectrum of the wild-type MaNiFB (Fig. 1c). These observations are exciting, as they provide strong support for the assignment of the respective Cys residues as the ligands of the three modules in MaNiFB while supplying direct proof for the [Fe₄S₄] identity of the K1- and K2-modules.
Probing the ligation patterns of NifB-associated clusters. The ligation patterns of the K1- and K2-modules were further explored by pulse EPR spectroscopy, using the one-dimensional electron spin echo envelope modulation (ESEEM) and two-dimensional hyperfine sub-level correlation (HYSCORE) pulse EPR techniques. In particular, we examined the presence of nitrogen coupling—previously reported for a NifB homolog from a different methanogenic organism27—in MaNifB<sup>31</sup> and MaNifB<sup>K2</sup> in order to assign the nitrogen ligand to a specific cluster module. Interestingly, the three-pulse ESEEM data of MaNifB<sup>wt</sup> and MaNifB<sup>K1</sup> reveal modulations of a nitrogen coupled to the K1-cluster (Fig. 2a, blue trace). Data collected across the EPR absorption envelope of MaNifB<sup>AM</sup> (Supplementary Fig. 5), from g = 2.050 to g = 1.900, show complex and overlapping peaks from a 14N nucleus. While these data are rich in information, extracting that information is difficult given the overlap between peaks. In order to further separate out the observed peaks and reliably interpret the 14N hyperfine and quadruple couplings, we collected HYSCORE spectra at each of the principal g values for the K1 module. The experimental data are simulated exceedingly well with a 14N hyperfine coupling tensor (in MHz) of A = [2.9 2.9 5.6] and a nuclear quadrupole coupling of $e^2Qq/h = -2.1$ MHz and $\eta = 0.4$ (Fig. 2b, c; also see Supplementary Table 3). The overall hyperfine coupling tensor, A, can be analyzed as two major components, where $A = A_{iso} + A_{dip}$. The first term ($A_{iso}$) is the isotropic Fermi contact term, where $A_{iso} = (A_1 + A_2 + A_3)/3$; and the second term ($A_{dip}$) is the through space dipolar coupling tensor, where $A_{dip} = [-T^T - T]$. We then compared known values of various N ligands to metal centers (Supplementary Table 3) with the HYSCORE simulation parameters. For a nitrogen directly coordinated to a FeS cluster, an isotopic hyperfine of $A_{iso} = 3–7$ MHz and a dipolar coupling of $T = 0.9–1$ MHz are expected, while a nitrogen which is near but not coordinated to the cluster, such as a backbone amide, would have an $A_{iso} \sim 1$ MHz. Our data suggest the nitrogen we measured ($A_{iso} = 3.8$ MHz, $T^{K1} = 0.9$ MHz) is in fact directly coordinated to the K1 module. Next, we compared the nuclear quadrupole coupling ($e^2Qq/h$) values of 14N nuclei, which report on the electric field gradient of the 14N nucleus and are sensitive to the environment of the nitrogen that is directly bound to an Fe center. While there are numerous EPR measured hyperfine and quadrupole couplings from nitrogenous ligands to Fe sites, a majority of them are from histidine ligated sites, including those found in the various Rieske-type [Fe<sub>2</sub>S<sub>2</sub>]

![Fig. 1](image-url) Assignment of three [Fe<sub>4</sub>S<sub>4</sub>] cluster modules in MaNifB. a. The primary sequence of MaNifB. The Cys and His residues that are conserved among 45 NifB sequences from various organisms<sup>35</sup> are highlighted, with those 100% conserved indicated with a *, and others noted for the percentages of conservation among these organisms. b. Schematic presentations of the SAM-, K1-, and K2-modules in MaNifB. A 3-Cys ligation pattern is proposed for all three modules, with the proposed ligands indicated in each module. c. EPR spectra of the synthetic [Fe<sub>4</sub>S<sub>4</sub>] cluster-reconstituted wild-type MaNifB (MaNifB<sup>wt</sup>, black) and MaNifB variants carrying the SAM (MaNifB<sup>SAM</sup>, brown), K1 (MaNifB<sup>K1</sup>, blue), and K2 (MaNifB<sup>K2</sup>, orange) modules, respectively. The spectra were collected as described in Methods, and the g values of each spectrum are indicated. The CW EPR experiment was performed four times. Representative results are shown in c. See Supplementary Table 1 for Fe contents, spin concentrations and protein concentrations of EPR samples.
clusters \((\varepsilon^2 Q_q h = 1.9-3.5 \text{ MHz})\) where the cluster contains two His ligands, in the mitoNEET \([\text{Fe}_4\text{S}_4]\) clusters \((\varepsilon^2 Q_q h = -2.47 \text{ MHz})\) which contain only a single His ligand, and in myoglobin \((\varepsilon^2 Q_q h = -2.24 \text{ MHz})\) where the His ligand is axial to the porphyrin-bound Fe center\(^{30-34}\). The only reported hyperfine and quadrupole coupling from a non-histidine ligand is found in the radical SAM enzyme BioB, where Arg\(^{260}\) is coordinated to a \([\text{Fe}_4\text{S}_4]\) cluster \((\varepsilon^2 Q_q h = 2.6-2.8 \text{ MHz})\)\(^{39}\). The quadrupole coupling we measured \((\varepsilon^2 Q_q h = -2.1 \text{ MHz})\) is lower than the reported value for Arg and is in the range of values for a His coordinated cluster; therefore, we assigned the fourth ligand to the K1 module as histidine. Contrary to what we observed in the case of the K1-cluster, we did not detect \(^{14}\text{N}\) coupling to the K2-cluster (in \(\text{MaNiB}^{K2}\)) and the L-cluster (in \(\text{MaNiB}^L\), or \(\text{SAM}-\text{treated MaNiB}^{\text{wt}}\), wherein the K1- and K2-modules are fused into an 8Fe L-cluster upon addition of SAM) by the same EPR techniques (Fig. 2a, red and green traces). However, the observed absence of nitrogen coupling to the K2 module cannot be used to determine if the fourth Fe site of this cluster is open, is occupied by a coordinated water, or is ligated by an amino acid such as Asp or Glu. The absence of nitrogen ligation to the L-cluster, on the other hand, is particularly interesting, as it suggests a conformational rearrangement upon coupling of the K1- and K2-modules into an 8Fe L-cluster.

**Defining the functions of NiFB-associated modules.** Having established the presence of three distinct \([\text{Fe}_4\text{S}_4]\) modules in \(\text{MaNiB}\), we set out to understand the question of which biosynthetic event occurs on each module and, particularly, how the SAM-module—the place where radical SAM chemistry takes place—works with the K1- and K2-modules during the cluster maturation process. Using our modular approach, two additional \(\text{MaNiB}\) variants (see Supplementary Fig. 2, iv, v), one carrying the SAM- and K1-modules (designated \(\text{MaNiB}^{\text{SAM+K1}}\)) and the other carrying the SAM- and K2-modules (designated \(\text{MaNiB}^{\text{SAM+K2}}\)), were expressed in \(E.\ coli\), followed by purification and reconstitution with the synthetic \([\text{Fe}_4\text{S}_4]\) compound. Interestingly, the spectrum of neither \(\text{MaNiB}^{\text{SAM+K1}}\) nor \(\text{MaNiB}^{\text{SAM+K2}}\) is a simple add-up of the individual spectra of the respective cluster modules: the \(\text{MaNiB}^{\text{SAM+K2}}\) spectrum resembles the \(\text{MaNiB}^{\text{SAM}}\) spectrum in line-shape, but is somewhat broadened and displayed a new \(g_{\text{app}} = 1.92\) feature (Fig. 3a, green trace); whereas the \(\text{MaNiB}^{\text{SAM+K1}}\) spectrum resembles the \(\text{MaNiB}^{\text{K1}}\) spectrum in line-shape, but is clearly broadened and displays a distinct \(g_{\text{app}} = 1.95\) feature in addition (Fig. 3a, blue trace). HPLC analysis further reveals that, like the wild-type \(\text{MaNiB}\), \(\text{MaNiB}^{\text{SAM+K2}}\) is capable of cleaving SAM into \(S\)-adenosyl-L-homocysteine (SAH) and 5'-deoxyadenosine (5'-dAH); in contrast, \(\text{MaNiB}^{\text{SAM+K1}}\), along with \(\text{MaNiB}^{\text{SAM}}\), \(\text{MaNiB}^{\text{K1}}\), and \(\text{MaNiB}^{\text{K2}}\), is unable to generate SAH or 5'-dAH upon incubation with SAM (Fig. 3b, c). This observation suggests that the presence of both SAM- and K2-modules is the prerequisite for both SAM-related reactions to occur during the cluster maturation process, with the K2 module serving as the final location for methyl attachment and the SAM-module supplying a 5'-dA radical for hydrogen abstraction of the K2-associated methyl group. Consistent with this suggestion, the formation of methanethiol is only detected upon acid quench of an incubation mixture of \(\text{MaNiB}^{\text{SAM+K2}}\) and SAM, pointing to the attachment of SAM-derived methyl group to a sulindle of the K2 module (Fig. 3d).

**Discussion**

A refined pathway of the K- to L-cluster conversion on NiFB (Fig. 4) can be proposed based on these results, as well as those...
from a recent work that traced the origin and insertion of the 9th sulfur of the cofactor. This pathway starts with methylene abstraction from one equivalent of SAM to a bridging sulfdie of the K2 module via an \( \text{S}_2\text{O}_2 \)-type mechanism, followed by generation of a 5′-dA radical via homolytic cleavage of a second equivalent of SAM bound to the SAM-module, and abstraction of a hydrogen atom from the K2-bound methyl group. These events result in a K2-bound methylene (−CH₂) radical, which then initiates the coupling/rearrangement of the K2- and K1-modules into an L*-cluster (\([\text{Fe}_8\text{S}_8\text{C}]\)) which, triggered by protonation, can be released from the central cavity of the cluster. Of the various reactions catalyzed by NiFB, the S₂O₂-type methyltransfer and the subsequent transfer of the L-cluster from NiFB onto the next module via an SN₂-type mechanism, followed by generation of a 5′-dAH MT radical and a catalytic [Fe₅S₄] cluster (i.e., the SAM-cluster). These [Fe₅S₄] clusters can be sensitive to degradation, especially when there is a non Cys-ligated, open Fe site. Therefore, it is advantageous to have a ligand (like the histidine ligand for the K1-cluster) which, triggered by protonation, can be released from the [Fe₅S₄] cluster module to facilitate the formation of the much more stable [Fe₅S₄C] L-cluster.

Another interesting point to note is the absence of 5′-dAH formation in the sole presence of the SAM-module, and the absence of SAH formation in the sole presence of the K2 module. While the former could be explained by a lack of K2-bound methyl group that can undergo hydrogen abstraction by a 5′-dA radical, the latter could be accounted for by the absence of a nearby SAM-module that renders the K2-cluster in the correct oxidation state for methyl attachment. A previous study has shown that methyltransfer does not occur when NiFB is oxidized or reduced by a weak reductant, suggesting that the K-cluster needs to be poised in a certain redox state to render its associated sulfdies more nucleophilic for methyltransfer via an SN₂-type nucleophilic substitution. It is possible, therefore, that the K2 module needs the presence of at least the SAM-module to be able to accept the methyl group, a scenario highlighting the importance of cross-talk between the cluster modules during the cluster transformation process. Additionally, the fact that the amounts of SAH and 5′-dAH formed in the absence of K1 module are much reduced compared to those in the presence of K1 module further points to a coordination between all three cluster modules to maximize the efficiency of cluster transformation.

While details of the various biosynthetic events on NiFB await further investigation, the current study conclusively establishes the presence of three [Fe₅S₄] units on NiFB and provides useful insights into the coordination and function of each module in the...
process of K- to L-cluster transformation, taking advantage of the synthetic [Fe8S9C] cluster-based reconstitution approach that can be applied to the functional analyses of a wide range of other FeS enzymes. The series of variants carrying different combinations of the three modules of NifB could be used to strategically uncouple the different steps in the cluster transformation process, and efforts along this line could be further combined with the identification and perturbation of the His ligand that provides the fourth N ligand to the K1 module and, possibly, one or more Cys ligands to both the K1- and K2-modules, to accommodate the structural rearrangement of the cluster during this process (iii, iv). This model is proposed based on data from this work and a related work28. The encircled black asterisk in the scheme of L*-cluster represents the vacant site in the belt region of the cluster, which may be occupied by a cysteine thiolate or water molecule.

Methods

General information. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo-Fisher Scientific (Waltham, MA), and all experiments were performed under an Ar atmosphere using Schlenk techniques and a glove box operating at <3 ppm O2.

Cell growth and protein purification. E. coli strains expressing His-tagged MnNifB95 (strain YM114EE), MnNifBSAM (strain YM163EE), MnNifB5 (strain YM163EE), MnNifB2 (strain YM166EE), MnNifB5SAM-K1 (strain YM180EE), and MnNifB5SAM-K2 (strain YM181EE) were grown in Difco LB medium containing 100 mg L−1 ampicillin (BD Biosciences) in a BIOFLO 415 fermenter (New Brunswick Scientific) at a temperature of 37 °C, an agitation of 200 rpm and an airflow of 10 L min−1. When cell density (measured at OD600) reached 0.5, 25 µM IPTG was added to the cell culture to induce protein expression at 25 °C for 16 h. Subsequently, cells were harvested by centrifugation using a Thermo-Fisher Scientific Legend XTR centrifuge, followed by purification of His-tagged MnNifB proteins using methods adapted from the purification of His-tagged nitrogenase proteins92.

Cluster reconstitution and maturation. The as-isolated wild-type or variant MnNifB protein was treated with 20 mM bathophenanthroline disulfonate, an iron chelator, in a buffer containing 5 mM MgATP, 2 mM dithionite (DT: Na2S2O4), 50 mM Tris–HCl (pH 8) and 500 mM NaCl, followed by incubation at room temperature for 1 h to remove the endogenous FeS clusters associated with the protein. Subsequently, this mixture was diluted with a buffer containing 50 mM Tris–HCl (pH 8) and loaded on a Q Sepharose column (GE Healthcare). The column was then washed with a buffer containing 2 mM DT, 50 mM Tris–HCl (pH 8) and 100 mM NaCl prior to elution of the MnNifB protein with a buffer containing 20 mM Tris–HCl (pH 8). Reconstitution of the wild-type or variant MnNifB protein with synthetic [Fe8S9C] clusters (designated [Fe8S9C]SAM) was carried out by adding a dimethylformamide (DMF) solution of [Fe8S9C]SAM dropwise at a molar ratio of 5:1 to the MnNifB protein in a buffer containing 2 mM DT, 20 mM β-mercaptoethanol, 50 mM Tris–HCl (pH 8), and 300 mM NaCl with continuous stirring on ice. After incubation on ice for 1 h, the reaction mixture was diluted with a buffer containing 2 mM DT and 50 mM Tris–HCl (pH 8) and loaded on a Q Sepharose column. The column was then washed with a buffer containing 2 mM

Fig. 4 Refined model of L-cluster formation on NifB. This process begins with methyltransfer from one SAM molecule to a sulfide of the K2 module via an Sβ2-type mechanism (i), and it is followed by the formation of a 5'-dA radical via homolytic cleavage of a second SAM molecule by the SAM module, and the subsequent hydrogen abstraction from the K2-bound methyl group by 5'-dA radical (ii). The resulting, K2-bound methylene (-CH2-) radical then initiates the coupling/rearrangement of the K2- and K1-modules into an L*-cluster ([Fe8S5C]) that resembles the L-cluster ([Fe8S5C]) except for the absence of a belt sulfur (i.e., the 9th sulfur) (iii). Subsequently, the 9th sulfur is inserted concomitantly with further deprotonation/dehydrogenation of the carbon intermediate until an interstitial C4= ion is generated in the center of a fully assembled L-cluster (iv). These events are accompanied by loss of a conserved His ligand to the K1 module and, possibly, one or more Cys ligands to both the K1- and K2-modules, to accommodate the structural rearrangement of the cluster during this process (iii, iv). This model is proposed based on data from this work and a related work28. The encircled black asterisk in the scheme of L*-cluster represents the vacant site in the belt region of the cluster, which may be occupied by a cysteine thiolate or water molecule.
DT, 50 mM Tris–HCl, and 100 mM NaCl to preequilibration of the reduced MoFe protein was subjected to a buffer containing 2 mM DT, 50 mM Tris–HCl (pH 8), and 500 mM NaCl. The pH was raised to 8 by the addition of 1 M HCl. The acid-quenched sample was then incubated at 60 °C for 15 min to release the volatile methanethiol into the headspace, followed by equilibration of the sample to room temperature for 10 min, and injection of the entire headspace by a gas-tight syringe onto a Restek Rxi-1ms column (30 m, 0.32 mm ID, 4 μm df) for analysis by GC–MS (Thermo-Fisher Scientific Trace 1300 GC connected to a Thermo-Fisher Scientific ISQ QD single quadrupole mass spectrometer). During each GC–MS run, the GC inlet and oven temperatures were kept at 30 °C, and the mass spectrometer transfer line and ion source were kept at 250 °C. Using SIM conditions in electron ionization mode, methanethiol was detected at an m/z ratio of 47.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the article and the Supplementary Information and from the corresponding authors upon reasonable request.

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Author contributions

L.A.R., J.W., C.C.L., M.T.S. and K.T. performed experiments and analyzed data; J.W., R.D.B. and Y.H. designed experiments, analyzed data, and wrote the manuscript.

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L.A.R., J.W., C.C.L., M.T.S. and K.T. performed experiments and analyzed data; J.W., R.D.B. and Y.H. designed experiments, analyzed data, and wrote the manuscript.

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