Identity and function of an essential nitrogen ligand of the nitrogenase cofactor biosynthesis protein NifB

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NifB is a radical S-adenosyl-L-methionine (SAM) enzyme that is essential for nitrogenase cofactor assembly. Previously, a nitrogen ligand was shown to be involved in coupling a pair of [Fe4S4] clusters (designated K1 and K2) concomitant with carbide insertion into an [Fe8S9C] cofactor core (designated L) on NifB. However, the identity and function of this ligand remain elusive. Here, we use combined mutagenesis and pulse electron paramagnetic resonance analyses to establish histidine-43 of Methanosarcina acetivorans NifB (MaNifB) as the nitrogen ligand for K1. Biochemical and continuous wave electron paramagnetic resonance data demonstrate the inability of MaNifB to serve as a source for cofactor maturation upon substitution of histidine-43 with alanine; whereas x-ray absorption spectroscopy/extended x-ray fine structure experiments further suggest formation of an intermediate that lacks the cofactor core arrangement in this MaNifB variant. These results point to dual functions of histidine-43 in structurally assisting the proper coupling between K1 and K2 and concurrently facilitating carbide formation via deprotonation of the initial carbon radical.
NiF/B plays a key role in the biosynthesis of the active site of Mo-nitrogenase, an [(R-homocitrate)-MoFe$_5$S$_4$C] metallocofactor (designated M-cluster) that catalyzes the ambient conversion of dinitrogen (N$_2$) to ammonia (NH$_3$) -2. A member of the radical S-adenosyl-L-methionine (SAM) enzyme family -3, 4, NiF/B is a monomeric protein carrying three [Fe$_4$S$_4$] clusters -5, 6, one, designated the SAM-cluster, is ligated by a canonical CxxxCxxC motif and coordinates the SAM-binding [Fe$_4$S$_4$] cluster; the other two, designated the K-cluster, are ligated by a number of conserved ligands and supply the full complement of iron (Fe) atoms for the biosynthesis of an 8Fe precursor to the mature cofactor. Previous studies of the NiF/B proteins from Azotobera vinelandii and Methanosarcina acetivorans have led to the proposal that NiF/B utilizes a unique, radical SAM-dependent mechanism for carbide insertion concomitant with the transformation of the K-cluster to an [Fe$_4$S$_4$C] cluster (designated the L-cluster), which requires both an 8Fe precursor and an all-Fe core of the M-cluster -7, 8, 9. This process begins with an S$_2$-2-type transfer of a metal group from a SAM molecule to the K-cluster (Supplementary Fig. 1a, ①), and it is followed by hydrogen abstraction of the methyl group by a 5'-deoxadenosyl (5'-dA) radical that is derived from the homolytic cleavage of a second SAM molecule (Supplementary Fig. 1a, ②). Once formed, the L-cluster is passed from Nif/B onto NifEN, the homologous protein of NifB (designated ifBN), and the resulting methylene radical then undergoes further deprotonation/dehydrogenation to yield a μ-coordinated interstitial carbide concomitant with the radical-chemistry-based coupling and rearrangement of the two [Fe$_4$S$_4$] modules of the K-cluster (designated K1- and K2-cluster, respectively) into an [Fe$_4$S$_4$C] L-cluster, which represents an all-Fe core of the M-cluster except for the substitution of one Fe atom for Mo/homocitrate at one end of the cluster (Supplementary Fig. 1a, ③). Once generated, the L-cluster is passed from NiF/B onto NiF/HN, the next biosynthetic scaffold, in which it is matured into a fully complemented M-cluster upon insertion of Mo and homocitrate by NiF/H prior to the delivery of M-cluster to its final binding site in NiF/DK, the catalytic component of Mo-nitrogenase (Supplementary Fig. 1a, ④) -11, 22.

The unique radical chemistry that occurs on NiF/B during the nitrogenase cofactor core assembly makes this protein an attractive subject of investigation, as knowledge in this regard will shed light on the unusual synthetic route to a high-nuclearity metallocofactor that is biologically important and chemically elusive. Previously, we have identified three sets of Cys ligands—three Cys per set—for the three [Fe$_4$S$_4$] clusters in M. acetivorans NiF/B (designated MaNiF/B), namely, the SAM- cluster (Cys$^{50}$, Cys$^{54}$, and Cys$^{57}$), the K1-cluster (Cys$^{50}$, Cys$^{63}$, and Cys$^{129}$) and the K2-cluster (Cys$^{264}$, Cys$^{274}$, and Cys$^{277}$) (Supplementary Fig. 1b, c) -11. Further, we have established the coexistence of SAM- and K2-clusters as a prerequisite for methyltransfer and hydrogen abstraction to occur and pinpointed the K2-cluster as the site for methyl attachment and the subsequent hydrogen abstraction from the methyl group by a 5'-dA radical -11. Perhaps even more excitingly, using pulse EPR techniques, we have identified a nitrogen atom from a His residue as the fourth ligand for the K1-cluster that is lost upon coupling between the K1- and K2-clusters into an L-cluster -11. This observation suggests an important role of the His/nitrogen ligand in generating the nitrogenase cofactor core (i.e., the L-cluster), particularly given the ability of His to undergo protonation/deprotonation, which either provides a release mechanism for the L-cluster onto the next biosynthetic apparatus or allows this residue to participate in the further deprotonation/dehydrogenation of the carbon radical to give rise to a central carbide. As such, it is crucial to identify this nitrogen ligand and probe its function in the process of nitrogenase cofactor core formation.

Here we use a combination of site-directed mutagenesis and pulse EPR spectroscopy to show that the His$^{43}$ residue is the specific nitrogen ligand for the K1-cluster of MaNiF/B. Our biochemical and EPR analyses demonstrate the essential role of His$^{43}$ in the formation of the nitrogenase cofactor core structure, although substitution of this residue with Ala does not impact early steps of carbide insertion leading to the initial hydrogen atom abstraction of the K2-associated methyl group. Our XAS/EXAFS analysis further reveals a shortened distance between the K1- and K2-clusters upon substitution of His$^{43}$ with Ala, as well as a further processing of these clusters into an intermediate between the K- and L-clusters upon incubation with SAM. These observations point to a dual function of His$^{43}$ in positioning/orienting the K1-cluster relative to the K2-cluster for proper coupling and deprotonating the initial carbon radical for carbide formation.

Results

The identity of the histidine ligand of the K1-cluster. Sequence analysis of MaNiF/B revealed the presence of three highly conserved His residues, His$^{28}$, His$^{43}$, and His$^{19}$, which could potentially serve as the nitrogen ligand for the K1-cluster on NiF/B (Supplementary Fig. 1b). On the basis of this analysis, three MaNiF/B variants were heterologously expressed in Escherichia coli. Designated MaNiF/B$^{H^{43}A}$, MaNiF/B$^{H^{43}A}$, and MaNiF/B$^{H^{219}A}$, respectively, each variant has one of the three conserved His residues substituted with Ala. Purified MaNiF/B variants, like their wildtype counterpart (designated MaNiF/B$^{WT}$), are monomers of ~38 kDa (Supplementary Fig. 2a).

Moreover, upon FeS reconstitution, MaNiF/B$^{H^{43}A}$, MaNiF/B$^{H^{43}A}$, and MaNiF/B$^{H^{219}A}$ have Fe contents comparable to that of MaNiF/B$^{WT}$, all of which carry three [Fe$_4$S$_4$] clusters (i.e., the SAM-, K1- and K2-clusters) per protein (Supplementary Fig. 2b). Thus, a loss of the His ligand does not seem to impact the ability of MaNiF/B to ligate any of the three [Fe$_4$S$_4$] clusters, likely due to the 3-Cys-coordination of these clusters that is sufficient to secure them in place.

To assess whether a nitrogen ligand is still present in these MaNiF/B variants, pulse EPR spectroscopy—specifically, three-pulse electron spin echo envelope modulation (3P-ESEEM)—was used to observe modulations to the time domain spectrum and corresponding peaks in the fast Fourier transformed (FT) spectrum that can be assigned to the coordinating nuclei (Fig. 1; also see Supplementary Fig. 3). Consistent with our previous observation, MaNiF/B$^{WT}$ shows deep modulations in the time domain of the ESEEM spectrum (Fig. 1a, trace 1) and corresponding intensity between 0 and 6 MHz in the FT (Fig. 1b, trace 1), which have been previously assigned to the hyperfine and quadrupole couplings of a cluster-ligated 1$^4$N nucleus -11. Similar modulations and intensities are present in the ESEEM spectrum and FT of MaNiF/B$^{K^{1}A}$, a variant carrying only the K1-cluster but no SAM- and K2-clusters (because of substitutions of the Cys ligands of the SAM- and K2-clusters with Ala) -11, confirming the previous assignment of the nitrogen ligand to the K1-cluster (Fig. 1a, trace 5). While similarly deep modulations and intensities are observed in the ESEEM spectra and FFTs of MaNiF/B$^{B^{28}A}$ and MaNiF/B$^{H^{219}A}$ (Fig. 1a, b, traces 2, 3), these features are clearly absent from the ESEEM spectrum and FT of MaNiF/B$^{H^{43}A}$ (Fig. 1a, b, trace 4), suggesting His$^{43}$ as the nitrogen ligand for the K1-cluster.

To seek further support for this assignment, MaNiF/B$^{K^{1}H^{43}A}$—another MaNiF/B variant carrying only the Cys ligands of K1 along with a substitution of His$^{43}$ with Ala—was heterologously expressed in E. coli. The purified MaNiF/B$^{K^{1}H^{43}A}$ demonstrates the same subunit composition as MaNiF/B$^{K^{1}}$, as well as the same Fe content that is consistent with the presence of one [Fe$_4$S$_4$] cluster (i.e., the K1-cluster) per protein upon FeS reconstitution (Supplementary Fig. 2a, b). However, contrary to MaNiF/B$^{K^{1}}$ (Fig. 1a, b, trace 5), MaNiF/B$^{K^{1}H^{43}A}$ does not show deep modulations and intensities in its ESEEM spectrum and FT (Fig. 1a, b, trace 6), firmly establishing His$^{43}$ as the nitrogen ligand that specifically coordinates the K1-cluster.
The role of histidine ligand in L-cluster maturation. Continuous wave (CW) EPR analysis provided the first insights into the function of His43 in the cofactor core assembly process. Consistent with the coupling and rearrangement of the K1- and K2-clusters into an L-cluster, an L-cluster-specific, \( g = 1.94 \) signal is observed in the spectra of MaNifBwt, MaNifBH28A, and MaNifBH219A upon incubation of these proteins with SAM (Fig. 2a, traces 1–3). In contrast, the \( g = 1.94 \) signal is absent from the spectrum of MaNifBH43A following the same treatment, suggesting a lack of K- to L-cluster transformation on this protein after incubation with SAM (Fig. 2a, trace 4). In support of this assignment, the SAM-treated MaNifBwt, MaNifBH28A, and MaNifBH219A can be used as M-cluster sources for the subsequent reconstitution and activation of apo-NifDK in an in vitro assay; whereas the SAM-treated MaNifBH43A cannot support the reconstitution and activation of apo-NifDK in the same assay (Fig. 2b). Interestingly, the activity of MaNifBH28A in this assay is ~41% less than those of MaNifBwt and MaNifBH219A, suggesting a possible involvement of His28 in the K- to L-cluster conversion due to the extremely close location of this residue to one of the Cys ligands (Cys30) of the K1-cluster in the primary sequence (see Supplementary Fig. 1b) and, consequently, the tertiary structure of NifB. More importantly, the abolished activity of in MaNifBH43A in the K- to L-cluster transformation points to a critical role of His43 in coupling the K1- and K2-clusters into an 8Fe L-cluster.

**Fig. 1 Three-pulse ESEEM spectra of dithionite-reduced MaNifB proteins.** a Time domain and (b) fast Fourier transformed (FFT) spectra of MaNifBwt (1), MaNifBH28A (2), MaNifBH219A (3), MaNifBH43A (4), MaNifBK1 (5) and MaNifBK1-H43A (6). The time domain spectra of the His-ligand containing samples (i.e., 1, 2, 3, and 5) have modulations from \(^{14}\)N that appear as peaks in the fast Fourier transformed (FFT) spectra between 1 and 6 MHz. The sharp modulations between 250 and 500 ns in the time domain and the resulting broad peak near 14 MHz in the FFT are from nearby weakly coupled protons. The ESEEM spectra were recorded at 10 K, \( \tau = 128-144 \) ns, \(\pi/2 = 12 \) ns, and 9.3366 GHz. The experiment was performed three times independently (n = 3 independent samples), and representative results are shown in the figure. All protein samples have a concentration of 15 mg mL\(^{-1}\). The CW EPR spectra of these dithionite-reduced MaNifB proteins are shown in Supplementary Fig. 3.

**Fig. 2 Conversion of L-cluster to M-cluster on MaNifB proteins.** a EPR spectra of IDS-oxidized MaNifBwt (1), MaNifBH28A (2), MaNifBH219A (3), and MaNifBH43A (4) upon addition of SAM. Formation of the L-cluster was monitored by the appearance of an L-cluster-specific \( S = 1/2 \) signal at \( g = 1.94 \) (dashed vertical line). All protein samples have a concentration of 15 mg mL\(^{-1}\). The EPR spectra were recorded at 5 mW and 20 K. b M-cluster maturation activity of MaNifBwt (1), MaNifBH28A (2), MaNifBH219A (3), and MaNifBH43A (4). The activity of M-cluster maturation was determined on the basis of the C\(_2\)H\(_2\)-reducing activity of reconstituted NifDK, using SAM-treated MaNifB proteins as the M-cluster sources. The EPR analysis was performed three times independently (n = 3 independent samples), and representative results are shown in (a). The maturation assay was performed five times independently (n = 5 independent samples), and data are presented as mean ± S.D. (b). See Methods for the detailed composition of maturation assays.
To further explore the role of His43 in this process, high performance liquid chromatography (HPLC) was performed to examine the products generated upon incubation of MaNifBH43A with SAM. Like MaNifBwt, MaNifBH28A and MaNifBH219A (Fig. 3a, b, traces 1–3), MaNifBH43A (Fig. 3a, b, trace 4) can cleave SAM into S-adenosyl-L-homocysteine (SAH) and 5′-deoxyadenosine (5′-dAH). In addition, as observed in the cases of MaNifBwt, MaNifBH28A, and MaNifBH219A (Fig. 3c, traces 1–3), formation of methanethiol is detected upon acid quench of an incubation mixture of MaNifBH43A and SAM (Fig. 3c, trace 4). This observation is not particularly surprising because our previous study demonstrates that SAH, 5′-dAH and methanethiol can be generated as long as both SAM- and K2-clusters are present, even in absence of the K1-cluster. The fact that substitution of the K1-specific ligand, His43, with Ala does not impact the reactivities associated with the SAM- and K2-clusters is consistent with this observation and places the perturbation of the K- to L-cluster conversion by this substitution after the hydrogen atom abstraction from the K2-associated methyl group (see Supplementary Fig. 1a).

Dual actions of histidine ligand in L-cluster maturation. X-ray absorption spectroscopy (XAS)/extended x-ray absorption fine structure (EXAFS) analysis provided further insights into the role of His43 in L-cluster maturation. XAS/EXAFS analysis has proven to be a valuable tool for obtaining structural information of the cluster species related to the function and assembly of nitrogenase, and previous studies of the wildtype and variant MaNifB proteins have established XAS/EXAFS parameters that can be used in combination with the EPR and biochemical data to conclusively assign cluster species and monitor cluster transformation in this protein. The x-ray absorption near edge structure (XANES) data reveal a K-edge energy for MaNifBH43A similar to that for MaNifBw before or after incubation with SAM (Fig. 4a), suggesting a similar sulfur-rich environment in all these protein species16. The pre-edge feature of MaNifBH43A is also similar in intensity to that of MaNifBw before incubation with SAM; however, its amplitude does not increase as much as that of MaNifBw upon incubation with SAM (Table 1), suggesting that the transition metal center in SAM-treated MaNifBH43A (designated MaNifBH43A/SAM) is less distorted away from centrosymmetry23,24 than that in SAM-treated MaNifBw (designated MaNifBw/SAM). Consistent with this observation, the smooth second derivative of the pre-edge data of MaNifBw/SAM transitions from a single inverted peak at ~7112.6 eV to two inverted peaks at ~7112.6 eV and ~7114.5 eV, respectively (Fig. 4b). Such a change has been attributed to the conversion of the K-cluster (with typical tetrahedral Fe-site geometries25) to an L-cluster (with an unusual intermediate geometry between tetrahedral and trigonal pyramidal25) in MaNifBw upon incubation with SAM16. In the case of MaNifBH43A, while a similar peak at ~7112.6 eV is observed in the second derivative before and after incubation with SAM, the line-shapes of these plots beyond 7113 eV are different than those of the corresponding MaNifBw species (Fig. 4b). More importantly, the second peak at ~7114.5 eV is absent from the plot of MaNifBH43A/SAM, although MaNifBH43A/SAM seems to undergo a transition analogous to that of MaNifBw/SAM on the basis of the similar line-shapes of their second derivative plots (Fig. 4b).

Extended X-ray absorption fine structure (EXAFS) analysis of the Fe K-edges of MaNifBH43A provided important insights into the structural metrics of its associated cluster species. Prior to SAM treatment, MaNifBH43A and MaNifBw display two similar features at R + Δ ~1.7 and 2.4 Å, respectively, in the Fourier transforms (FT; Fig. 4c) of their EXAFS data (Fig. 4d), although the feature of MaNifBH43A at R + Δ ~2.6 Å is much more prominent than that of MaNifBw. For MaNifBH43A, these FT features can be best fit with Fe–S and Fe–Fe scatterers at 2.29 and 2.71 Å, respectively; whereas for MaNifBw, they are best fit with Fe–S scatterers at 2.29 Å and Fe–Fe scatterers at 2.51 and 2.69 Å, respectively (Table 2). Upon incubation with SAM, MaNifBH43A/SAM and MaNifBw/SAM seemingly undergo similar changes, both showing extra FT features at R + Δ ~3.0 and ~3.5 Å; yet, while MaNifBw/SAM displays a substantially increased intensity and a clear shift of its FT feature at R + Δ ~2.4 Å, the corresponding FT feature of MaNifBH43A/SAM remains largely unchanged (Fig. 4c). The differences between the two SAM-treated MaNifB species are clearly illustrated in the best fits of their EXAFS data: MaNifBH43A/SAM is best modeled with two types of Fe–S scatterers at 2.27 and 3.88 Å, respectively, and one type of Fe–Fe scatterers at 2.69 Å; whereas MaNifBw/SAM is best modeled with one type of Fe–S scatterers at 2.23 Å and two types of Fe–Fe scatterers at 2.64 and 3.70 Å, respectively (Table 2). Most notably, the long-range Fe–Fe distance at 3.70 Å, which originates from the intercubane scattering between the six carbide-coordinated
Fe atoms at the cofactor core\textsuperscript{25}, is present only in \textit{MaNifB}\textsuperscript{wt}/SAM but absent from \textit{MaNifB}\textsuperscript{H43A}/SAM. This observation suggests that unlike \textit{MaNifB}\textsuperscript{wt}, \textit{MaNifB}\textsuperscript{H43A} does not enable the formation of an L-cluster with a μ₆-coordinated central carbide in place upon incubation with SAM. In support of this argument, \textit{MaNifB}\textsuperscript{H43A} carries clusters with short-range Fe–Fe distances that are characteristic of the [Fe₄S₄] clusters before and after incubation with SAM (Table 2). However, modeling of the cluster species on \textit{MaNifB}\textsuperscript{H43A}/SAM, contrary to that of the cluster species on \textit{MaNifB}\textsuperscript{wt}, requires the inclusion of an extra Fe–S distance at 3.88 Å (Table 2) that corresponds to the distance from a sulfide to a Fe at the opposite vertex of a [Fe₄S₄] cluster. The appearance of such a distance is consistent with an increased order of the two K-cluster units (i.e., K₁ and K₂), or a further processing of these [Fe₄S₄] units into a cluster intermediate between the K- and L-clusters on \textit{MaNifB}\textsuperscript{H43A}/SAM; more importantly, it highlights an overall homogeneity of the cluster species on \textit{MaNifB}\textsuperscript{H43A}/SAM, as this Fe–S distance can only be observed when the clusters are well-ordered and, therefore, uniform in nature. Overall, the mean squared deviations (σ²) of the S and Fe scatterers are rather small (<5 × 10⁻³ Å²; see Supplementary Tables 1 and 2), which further supports the homogeneity of the cluster species in the \textit{MaNifB} proteins. Three most likely configurations can be proposed for this cluster intermediate: (i) a partial [Fe₄S₄] cluster pair bridged by two sulfide (S²⁻) atoms and a carbon (CH₃) species, (ii) a face-on [Fe₄S₄] cluster pair bridged by a CH₂ species, and (iii) a vertex-on [Fe₄S₄] cluster pair bridged by a CH₂ species, all of which lack the characteristic cofactor core structure that is defined by an interstitial carbide coordinated with six Fe atoms (Supplementary Fig. 4). Given the homogeneity of the cluster species on \textit{MaNifB}\textsuperscript{H43A}/SAM, it is likely that one of these proposed models will be identified through future structural characterization of this protein.

### Discussion

Formation of a cluster intermediate on the SAM-treated \textit{MaNifB}\textsuperscript{H43A} that is distinct from both the K- and L-clusters implies that His\textsuperscript{43} serves as a key structural element and/or reaction component during the process of cofactor core formation on \textit{MaNifB}. The substantially increased intensity of the FT feature of \textit{MaNifB}\textsuperscript{H43A} at R + Δ ~ 2.4 Å relative to that in \textit{MaNifB}\textsuperscript{wt} prior to incubation with SAM (see Fig. 4c) points to a much stronger Fe–Fe scattering that results from a much closer distance between the two [Fe₄S₄] units of the K-cluster in \textit{MaNifB}\textsuperscript{H43A} than those in \textit{MaNifB}\textsuperscript{wt}. Such a change in the distance and/or orientation of the K1-cluster relative to that of the K2-cluster apparently presents a challenge for \textit{MaNifB}\textsuperscript{H43A} to initiate a proper coupling between the two K-cluster units. The His\textsuperscript{43} ligand, therefore, could play a steric role in keeping the two K-cluster units in the correct distance/orientation to each other by either indirectly pulling the K1-cluster away from the K2-cluster via its ligand capacity or directly separating the K1- and K2-clusters with its bulky imidazole ring (Supplementary Fig. 4, ①). Additionally,
given our observation of a loss of the nitrogen ligand upon conversion of the K-cluster to an L-cluster on MaNiF, His43 may lose its coordination to the K1-cluster via protonation, thereby freeing up K1 for the subsequent coupling with K2 into an L-cluster, and facilitating the release of the completed L-cluster to the next biosynthetic apparatus for further maturation. As such, His43 likely functions as a molecular switch via reversible protonation/deprotonation events, securing the cluster in place in its deprotonated state while giving the cluster certain structural flexibility in its protonated state to accommodate the different states required for cluster conversion. A similarly labile nitrogen ligand is also found in mitoNEET, where protonation of different states required for cluster conversion.

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.

Table 2 Best fits of the Fe K-edge EXAFS data.

| Proteina | Fe-S | Fe-S-S | Fe-S-S-S | Fe-S-S-S-S |
|----------|------|--------|----------|------------|
|          | N    | R(Å)  | σ² (10⁻³) | N          | R(Å)  | σ² (10⁻³) | N          | R(Å)  | σ² (10⁻³) |
| MaNiFwt  | 3.8  | 2.29  | 8.19     | 1          | 2.51  | 5.82     | 1.5        | 2.69  | 4.35     |
| MaNiFwt/SAM | 3.1  | 2.23  | 4.23     | 3.5        | 2.64  | 7.87     | 1.5        | 3.70  | 7.89     |
| MaNiFwt/H43A | 3    | 2.29  | 2.98     | 2          | 2.71  | 4.02     | -          | -     | -        |
| MaNiFwt/H43A/SAM | 3    | 2.27  | 4.18     | 2          | 2.69  | 4.19     | -          | -     | -        |
| MaNiFwt/H43A | 3    | 3.88  | 15.3     | -          | -     | -        | -          | -     | -        |

*Data is fit for k = 2–11.2 Å⁻¹. Data for MaNiFwt and MaNiFwt/H43A/SAM are taken from Ref. 16. See Supplementary Tables 1 and 2 for details of EXAFS data fits for MaNiFwt/H43A and MaNiFwt/H43A/SAM, respectively.

Cell growth and protein purification. E. coli strains expressing His-tagged MaNiFwt (strain YM114EE), MaNiFwt/H43A (strain YM242EE), MaNiFwt/H28A (strain YM244EE), MaNiFwt/H43A/SAM, and MaNiFwt/K1-11-14-34 (strain YM307EE) were grown in 10-L batches in Difco LB medium containing 100 mg L⁻¹ ampicillin (BD Biosciences) in a BIOFLO 415 fermenter (New Brunswick Scientific) at 37 °C, with 200 rpm agitation and 10 L min⁻¹ airflow. When OD₅₉₅ reached 0.5, the temperature was lowered to 25 °C before expression of the wildtype and variant MaNiF proteins was induced by addition of 25 µM IPTG. Expression of proteins was allowed to continue for 16 h before cells were harvested by centrifugation using a Thermo Fisher Scientific Legend XTR centrifuge. Subsequently, His-tagged MaNiF proteins were purified by immobilized metal affinity chromatography (IMAC).67

SDS-PAGE analysis. The purified wildtype and variant MaNiF proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 4–20% precast Tris-glycine gel (Bio-Rad).

Iron determination. The iron contents of the Fe₄S₄-reconstituted wildtype and variant MaNiF proteins were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Thermo Scientific ICAP7000. Stock solutions of elemental iron (1 mg mL⁻¹, Inorganic Ventures) were diluted to make standard solutions for calibration. Each protein sample was mixed with 100 µL concentrated sulfuric acid (H₂SO₄) and 100 µL concentrated nitric acid (HNO₃) and heated at 250 °C for 30 min. This procedure was repeated until the solutions became colorless. The solution was then cooled to room temperature and diluted to a total volume of 10 mL with 2% HNO₃ prior to sample analysis.

Fe₄S₄ cluster reconstitution. The purified wildtype or variant MaNiF was treated with 20 mM bathophenanthroline disulfonate, an iron chelator, in a buffer containing 2 mM dithionite (DT; Na₂S₂O₄), 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl, followed by incubation at room temperature for 1 h to remove the endogenous Fe₄S₄ clusters associated with the protein. Subsequently, this mixture was diluted with a buffer containing 50 mM Tris-HCl (pH 8.0) 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 8.0) and 50 mM NaCl prior to elution of the MaNiF protein with a buffer containing 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. Dithionite was removed by running protein through a Sephadex G-25 (GE Healthcare) column equilibrated with 50 mM Tris-HCl (pH 8.0) and 10% glycerol. Reconstitution of the wildtype or variant MaNiF with synthetic [Fe₄S₄] clusters was carried out by adding a dimethylformamide (DMF) solution of synthetic [Fe₄S₄] cluster dropwise at a molar ratio of 5:1 to the MaNiF protein in a buffer containing 20 mM β-mercaptoethanol and 50 mM Tris-HCl (pH 8.0), 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 8.0) and loaded on a Q Sepharose column. The column was then washed with a buffer containing 2 mM DT, 50 mM Tris-HCl and 50 mM NaCl prior to elution of the reconstituted MaNiF with a buffer containing 2 mM DT, 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. Dithionite was removed by running protein through a Sephadex G-25 (GE Healthcare) column equilibrated with 50 mM Tris-HCl (pH 8.0) and 10% glycerol. Reconstituted wildtype and variant MaNiF proteins were subjected to metal determination, activity assays and EPR analysis.

Cofactor maturation assays. The cofactor maturation assay contained, in a total volume of 1.0 mL, 25 mM Tris-HCl (pH 8.0), 20 mM DT, 3.5 mg Fe₄S₄-reconstituted wildtype or variant MaNiF, 10 mM SAM, 2 mg ΔNiFΔαNiEN, 1.4 mg NiH, 0.8 mM ATP, 1.6 mM MgCl₂, 10 mM creatine phosphate, 8 units creatine phosphokinase, 0.3 mM homocitrullate, 0.3 mM Na₃MoO₄, and 0.5 mg ΔNiFΔαNiDK. This mixture was incubated at 30 °C for 30 min before it was examined for enzymatic activities.6,10

S-adenosyl-L-methionine (SAM) cleavage assays. The SAM cleavage assay contained, in a total volume of 0.3 mL, 25 mM Tris-HCl (pH 8.0), 5% glycerol (v/v), 40 µM wildtype or variant MaNiF, and 0.3 mM SAM. Assays were incubated...
at 25 °C for 60 min with intermittent mixing, before they were terminated by filtration through Amicon Ultra 30,000 MWCO centrifugal filters. Samples were then stored at 4 °C until analyzed by HPLC (silica gel reversed-phase, C-18, before being analyzed by a Thermo Scientific DionexUltimate 3000 UHPLC system equipped with an Acclaim 120 C18 column (4.6 × 100 mm, 5-µm particle size). The flow rate of buffer was 0.5 mL·min⁻¹ and the column was kept at 30 °C. The column was equilibrated with 98% buffer A (50 mM KH₂PO₄, pH 6.6) and 2% buffer B (100% methanol) before each injection of a 100-µL sample. After sample injection, a linear gradient of 2–60% buffer B was applied over 20 min, followed by 8 min of isocratic flow with 60% buffer B and a linear gradient of 60–2% buffer B over 4 min. Elution of products was monitored at a UV wavelength of 254 nm. After each run, the column was equilibrated for 5 min with 2% buffer B before the injection of the next sample.

**Acid quench experiments.** Detection of MnNiB-dependent production of methanethiol was performed.¹⁴ First, excess DT was removed from wildtype and variant MnNiB via gel filtration with Sephadex G-25 fine resin that was equilibrated with a buffer containing 25 mM Tris-HCl (pH 8.0). Immediately following the removal of excess reagent, 40 nmol of MnNiB was added to a sealed 300-µL glass vial that contained 400 nmol SAM in a total volume of 100 µL. These 100-µL reaction mixtures were then injected into a column containing MnNiB and were being quenched by 25 µL of 1 M HCl. To observe the formation of the volatile methanethiol, the acid-quenched samples were incubated at 60 °C for 15 min and equilibrated to room temperature for 10 min before the entire headspace was injected by a gas-tight syringe onto a GC–MS (Thermo Fisher Scientific Trace 1300 GC connected to a Thermo Scientific ISQ QD single quadrupole mass spectrometer) with a Restek Rxi-1ms column (30 m, 0.32 mm ID, 0.4 µm df). The GC inlet and oven temperatures were maintained at 30 °C, while the mass spectrometer transfer line and ion source were maintained at 250 °C. Total ion chromatograms were generated under UMD conditions in electron ionization mode, and methanethiol was detected at an m/z ratio of 47. The base peaks were selected on the basis of the characterization of standard samples (Sigma-Aldrich) under full scan conditions and comparison to those reported in the National Institute of Standards and Technology database.

**Electron paramagnetic resonance (EPR) analysis.** Sample preparation was carried out in a Vacuum Atmospheres glove box with less than 1 ppm O₂ and 2% flash frozen in liquid nitrogen before analysis. The dithionite-reduced samples were prepared by mixing the wildtype or variant MnNiB with 40 mM SAM for 15 min, followed by re-isolation of MnNiB into a buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 2 mM dithionite (DT). The indigo disulfonate (IDS)-oxidized samples were prepared by incubating the re-isolated MnNiB with excess IDS for 5 min, followed by removal of excess IDS which was using a Sephadex G-25 desalting column. The DT-reduced or IDS-oxidized MnNiB samples were subjected to 16 ns steps. ESEEM spectra were recorded at 10 K, following a modified data analysis protocol,²⁵ the Fe-K-edge EXAFS data for the clusters associated with MnNiB⁴¹⁻³⁴⁻ and MnNiB⁴⁻⁴⁴⁻/SAM were generated by subtracting the k-weighted EXAFS data, χ(κ), of MnNiB⁴⁻⁴⁴⁻ (an MnNiB variant carrying only the SAM-cluster) from the χ(κ) of the samples in a 1:2 ratio on the basis of the proportionate iron quantity for each cluster species (i.e., 4 Fe for the SAM-cluster and 8 Fe for the K-clusters). Theoretical phase and amplitude parameters for a given absorber–scatterer pair were calculated using FEFF 8.0 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 NiFeD (PDB code 3U7Q), where the Mo atom was exchanged for an Fe atom, or from the [Fe₅S₄]Cl₃ cluster in NiFe (PDB code 1GSP), because there are no available crystal structures of MnNiB. 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 lengths (R) and mean-square deviation (σ²) were allowed to freely float. The estimated uncertainties in R, σ², and N are 0.02 Å, 0.1 × 10⁻³ Å², and 20%, respectively. The amplitude reduction factor S₀ was fixed at 1.0 for the Fe K-edge data, whereas the edge-shift parameter ΔE₀ 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 × number of shells + 1. The goodness of fit (GOF) parameters were calculated as follows:

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

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

The Fe K-edge data were analyzed with a k range of 2–11.2 Å⁻¹ (ΔR = 0.17 Å) to allow comparison between previously reported data, although the data could be analyzed with higher resolution with a k range of 2–14 Å⁻¹ for MnNiB⁴⁻⁴⁻ and MnNiB⁴⁻⁴⁻/SAM. Pre-edge analysis was performed on the Fe K-edge fluorescence data normalized to have an edge jump of 1.0 at 7130 eV in PYPYLINE. The pre-edge features were fitted as described elsewhere (k) between 7188 and 7117 eV using the Fityk program with pseudo-Voigt functions composed of 50:50 Gaussian: Lorentzian functions.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding authors upon reasonable request. The National Institute of Standards and Technology database is available at https://www.nist.gov/data. Parameters used for cluster modeling are available at https://www.rcsb.org/ using PDB IDs 3U7Q and 1GSP.

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**Author contributions**

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

**Competing interests**

The authors declare no competing interests.

**Additional information**

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