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Biosynthesis of Nitrogenase Metalloclusters

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1. INTRODUCTION

Nitrogenase is a complex metalloenzyme that is best known for its function in biological nitrogen fixation.^{1,2} Harbored in a group of microbes called diazotrophs, nitrogenase catalyzes the reduction of nitrogen (N₂) to ammonia (NH₃) in a reaction that is usually depicted as $N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$. This reaction not only represents a key step in the global nitrogen cycle, but also embodies the formidable chemistry of breaking the exceptionally stable N≡N triple bond. Recently, nitrogenase was shown to reduce carbon monoxide (CO) to hydrocarbons under the same reaction conditions of biological nitrogen fixation,^{3–6} defining it as a versatile metalloenzyme that is capable of activating N₂ and CO and converting them into products of agronomic and economic values. Interestingly, the reactions of N₂- and CO-reduction by nitrogenase parallel two important processes in industry: the Haber-Bosch process, which is used for ammonia production from N₂ and hydrogen (H₂);⁷ and the Fischer-Tropsch process, which is used for carbon fuel production from CO and H₂.⁸ However, contrary to the industrial processes, the nitrogenase-catalyzed reactions occur under ambient conditions, making this enzyme a fascinating subject from the perspective of chemical energy.

Three homologous nitrogenases, namely, the molybdenum (Mo), vanadium (V) and iron (Fe)-only nitrogenases, have been identified to date.^{9,10} The best studied among them is the Mo nitrogenase from *Azotobacter vinelandii*, which consists of two component proteins. One, designated the Fe protein (NifH), is a γ_2 -dimer that contains a subunit-bridging [Fe₄S₄] cluster per dimer and an ATP binding site within each subunit. The other, designated the MoFe protein (NifDK), is an $\alpha_2\beta_2$ -tetramer that contains two complex metalloclusters per $\alpha\beta$ -dimer: a P-cluster ([Fe₈S₇]) at the α/β -subunit interface and an M-cluster ([MoFe₇S₉C-homocitrate]) within the α -subunit.^{11–14} Catalysis by the Mo nitrogenase involves the formation of a complex between NifH and NifDK^{15,16} and the inter-protein transfer of electrons from the [Fe₄S₄] cluster of NifH, via the P-cluster, to the

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M-cluster of NifDK, where substrate reduction eventually occurs (Figure 1). Such an electron pathway highlights the functions of the P- and M-clusters in substrate reduction. Both are high-nuclearity metaloclusters with unusual structures not recognized in other biological systems, and both have evaded successful chemical synthesis so far. The unique properties of the P- and M-clusters of nitrogenase will be discussed below (section 1.1), followed by an overview of proteins involved in the biosynthesis of these clusters (section 1.2).

1.1. Properties of the metal clusters in nitrogenase

The P-cluster is bridged between the α - and β -subunits of NifDK at a position that is 10 Å below the surface of the protein.^{11–13} Structurally, it can be viewed as two $[\text{Fe}_4\text{S}_3]$ partial cubanes bridged by a μ_6 -sulfide (Figure 2A and B); whereas chemically, it can exist in three oxidation states (designated the P^{N} , P^{1+} and P^{OX} state, respectively). In the presence of excess dithionite, the P-cluster exists in an all-ferrous, diamagnetic state (designated the P^{N} -cluster). Following the treatment of a dye oxidant [*e.g.*, indigodisulfonate (IDS)], the P^{N} -cluster can be two-electron oxidized to a stable $S = \text{integer}$ (3 or 4) state (designated the P^{OX} -cluster), which displays a characteristic, parallel-mode electron paramagnetic resonance (EPR) signal at $g = 11.8$.^{17–19} Both the P^{N} - and the P^{OX} -clusters (Figure 2A and B) are covalently coordinated by six cysteinyl ligands in NifDK, three from the α -subunit ($\text{Cys}^{\alpha 62}$, $\text{Cys}^{\alpha 88}$ and $\text{Cys}^{\alpha 154}$) and three from the β -subunit ($\text{Cys}^{\beta 70}$, $\text{Cys}^{\beta 95}$ and $\text{Cys}^{\beta 153}$). Each of the $\text{Cys}^{\alpha 62}$, $\text{Cys}^{\alpha 154}$, $\text{Cys}^{\beta 70}$ and $\text{Cys}^{\beta 153}$ ligands coordinates one Fe atom, and each of the $\text{Cys}^{\alpha 88}$ and $\text{Cys}^{\beta 95}$ ligands coordinates two Fe atoms of the P-cluster.^{20,21} However, the core structures of the P^{N} - and P^{OX} -clusters are different, with one half of the P^{OX} -cluster present in a more open conformation (Figure 2B). Such a structural rearrangement is accompanied by a change in the ligation pattern, as the P^{OX} -cluster is coordinated by two more protein ligands than the P^{N} -cluster.²¹ One of these ligands is $\text{Ser}^{\beta 188}$, which coordinates an Fe atom through an O γ ligand together with the cysteinyl group of $\text{Cys}^{\beta 153}$; the other ligand is $\text{Cys}^{\alpha 88}$, which coordinates an Fe atom through a backbone amide nitrogen ligand and a cysteinyl group (Figure 2B).

The M-cluster (also called FeMoco or cofactor) is buried within the α -subunit of NifDK, 14 Å away from the P-cluster. Structurally, the M-cluster can be viewed as $[\text{Fe}_4\text{S}_3]$ and $[\text{MoFe}_3\text{S}_3]$ partial cubanes bridged by three μ_2 -sulfides (Figure 2C). In addition to its metal-sulfur core, the M-cluster also contains an organic homocitrate moiety attached through its 2-hydroxy and 2-carboxyl groups to the Mo atom and a μ_6 -interstitial carbide coordinated in the central cavity.^{11–14} The interstitial carbide cannot be exchanged upon turnover, nor can it be used as a substrate and incorporated into the products, suggesting a role of the interstitial carbide in stabilizing the structure of the M-cluster.^{22,23} However, a function of this atom in indirectly modulating the reactivity of the M-cluster or directly interacting with the substrate cannot be excluded.²⁴ The M-cluster is coordinated by only two ligands in NifDK: $\text{Cys}^{\alpha 275}$, which coordinates the terminal Fe atom; and $\text{His}^{\alpha 442}$, which coordinates the opposite Mo atom. A third residue, $\text{Lys}^{\alpha 426}$, provides an additional hydrogen-bonded anchor for homocitrate at the Mo end of the cluster.^{11–14} In addition to the covalent ligands, the M-cluster is held within NifDK through direct and water-bridged hydrogen bonds. The apparently “simple” coordination pattern of the M-cluster permits extraction of this cluster

as an intact entity into organic solvents, such as N-methylformamide (NMF).^{25–27} The extracted M-cluster was shown to be anionic²⁶ despite a proposed charge of +1 or +3 for the metal-sulfur core of this cluster in the resting state.^{28,29} The overall negative charge of the M-cluster is believed to originate from its endogenous homocitrate entity, which is –4 if the hydroxyl (-OH) group is deprotonated. The extracted M-cluster can bind CO and cyanide (CN[–]) at certain oxidation states.^{30,31} Moreover, it can catalyze the ATP-independent reduction of CO and CN[–] to hydrocarbons in the presence of a strong reductant, europium(II) diethylenetriaminepentaacetate [Eu(II) DTPA],³² although conditions are yet to be defined for N₂ reduction by the extracted M-cluster. Both the solvent-extracted and the protein-bound M-clusters display a characteristic, $S = 3/2$ EPR signal at $g = 4.7$, 3.7 and 2.0 in the presence of excess dithionite; however, the signal displayed by the extracted M-cluster is broader in line-shape than that displayed by its protein-bound counterpart.^{26,33} Moreover, the M-cluster can undergo a reversible one-electron oxidation and reduction process, which is reflected by the disappearance of the $S = 3/2$ signal upon oxidation and the re-appearance of this signal upon re-reduction.¹

1.2. Roles of *nif* gene products in cluster biosynthesis

The P- and M-clusters are the most complex metalloclusters found in Nature. Consequently, the biosynthetic pathways of both clusters are highly complex and involve the actions of multiple nitrogen fixing (*nif*) gene products (please see Table 1 for the designations and assigned functions of *nif* gene products).^{34–39} The two pathways share two common apparatus, NifS and NifU, at the early stage of assembly, where small FeS clusters are generated as building blocks for the further construction of both P- and M-clusters.⁴⁰ NifS and NifU have complementary functions in mobilizing Fe and S for the formation of small FeS fragments: the former is a pyridoxal-dependent cysteine desulfurase, which is responsible for the formation of a protein-bound cysteine persulfide; whereas the latter is a scaffold protein, which receives the sulfur intermediate from the former for the sequential formation of [Fe₂S₂] and [Fe₄S₄] clusters.^{41–46} The biosynthesis of the two nitrogenase clusters are believed to branch at this point and, in the case of P-cluster, the assembly continues on NifDK, which receives the [Fe₄S₄] units from NifU and processes it into an [Fe₈S₇] P-cluster. This process requires the action of NifH, as deletion of *nifH* results in a form of NifDK that contains a precursor form to the mature P-cluster.^{47–49} It also requires the action of NifZ, as deletion of *nifZ* results in a form of NifDK that contains only 50% of the P-cluster content of the wild-type protein.^{50–52} Recent studies have established an essential role of NifH in coupling two 4Fe subclusters into an 8Fe P-cluster, as well as a critical role of NifZ in the stepwise assembly of the two P-clusters in NifDK, defining these two proteins as the key factors for the biosynthesis of P-cluster.

Continuation of the M-cluster assembly, on the other hand, is a lot more complicated from the “4Fe” branching point. A number of *nif* gene products have been proposed to function in this process, which consists of three major biosynthetic events: (1) formation of a large FeS core; (2) attachment of Mo and homocitrate to the cluster; and (3) transfer of the cluster between assembly sites. The first event is believed to occur on a scaffold protein called NifB. The essential function of this protein in M-cluster assembly was demonstrated by the accumulation of an M-cluster-deficient, apo form of NifDK in the cell upon deletion of

nifB.^{53–56} Moreover, it was shown that an “FeS core” of unknown composition (termed NifB-co) could be isolated from detergent-treated membranes and added to extracts of *nifB*-deletion strains to achieve activation of the apo NifDK in the presence of homocitrate, Mo and MgATP.^{57,58} The NifB-co was later extracted onto NifX, an “escort” protein proposed for cluster transfer during the process of M-cluster assembly, and modeled as a 6Fe analog of the M-cluster based on the best fits of EXAFS and NRVs data, suggesting that NifB synthesizes a fragment of the M-cluster that needs to be fused to another fragment at a later step in the assembly process.^{59,60} However, such a proposal was refuted by a recent study showing that two 4Fe clusters could be coupled into an 8Fe core of the M-cluster on NifB concomitant with SAM-dependent insertion of an interstitial carbide atom.^{61–63} Identification of this 8Fe cluster not only established a key role of NifB in synthesizing the complete core structure of the M-cluster, but also placed the event of Mo and homocitrate attachment at a later step than NifB.

This event is believed to occur on NifEN, another essential scaffold protein for M-cluster biosynthesis.^{53,54} NifEN can complement the function of NifB in M-cluster assembly, as the inactive crude extracts of *nifH*- and *nifB*-deletion strains could be mixed to produce an extract with nitrogenase activity.⁶⁴ In addition, NifH was shown to be interacting with NifEN in an unknown function related to this process, with one study proposing NifH as a carrier of a Mo-containing M-cluster precursor.^{65–73} Recent studies have demonstrated that the 8Fe core generated by NifB can indeed be matured into an M-cluster on NifEN upon insertion of Mo and homocitrate by NifH, defining NifEN as an M-cluster maturase and NifH as an ATP-dependent Mo/homocitrate insertase.^{74,75} The formation of homocitrate, which involves the condensation of acetyl-CoA and α -ketoglutarate, was shown to be catalyzed by NifV;⁷⁶ the mobilization of Mo prior to the involvement of NifH, however, is not well understood, except that the biosyntheses of M-cluster and molybdopterins share a common Mo transport pathway.⁷⁷ Based on EPR data, it was suggested that NifQ, which contains a number of Cys residues at certain spacing, might carry a [MoFe₃S₄] cluster.⁷⁸ However, it is unlikely that such a cluster could be an obligate Mo donor or a relevant precursor to M-cluster, because *nifQ*-deletion strains can still form M-clusters when Mo or cysteine is supplemented.^{79,80} Thus, instead of having a specific role in M-cluster biosynthesis, NifQ may have a more general function in Mo storage.

The event of cluster transfer between the assembly sites occurs at two stages of M-cluster biosynthesis. One involves the transfer of cluster from NifB to NifEN; whereas the other involves the transfer of cluster from NifEN to NifDK. Three “escort” proteins, NifX, NifY and NafY, have been proposed to function in these steps and deliver the precursor or the M-cluster from one assembly site to another. This proposal was based on the sequence homology of NifX, NifY and NafY to the carboxyl end of NifB, the respective locations of the genes encoding NifX and NifY in the same operons as those encoding NifEN and NifDK, and the abilities of NifX and NafY to bind NifB-co and M-cluster.^{81–83} However, none of the three proteins is essential for M-cluster biosynthesis, as single-, double- and triple-deletions of their encoding genes showed no impact on the abilities of these strains to fix nitrogen.^{84,85} Recently, it was shown that the M-cluster could be transferred from NifEN to NifDK via direct protein-protein interaction, precluding the absolute requirement for an

“escort” protein in this process.^{74,75} A direct cluster transfer from NifB to NifEN can also be proposed based on the presence of a naturally occurring NifEN-B fusion protein in *Clostridium pasteurianum* and the success in duplicating such a fusion between NifB and NifEN in *A. vinelandii*.⁶¹ These results point to a plausible “housekeeping” function of the proposed “escort” proteins, one that is involved in the scavenging of “free” FeS clusters related to M-cluster assembly. Alternatively, these partial fragments of NifB could originate from a duplication of the *nifB* gene within the *nif* gene cluster (*i.e.*, *nifX* and *nifY*) or elsewhere in the genome (*i.e.*, *nafY*) and the subsequent deletion/mutation of these duplicated genes during evolution that rendered their gene products no longer indispensable for M-cluster assembly.

The categorization of various *nif* gene products as essential and non-essential proteins for the biosyntheses of P- and M-clusters has proven to be crucial for identifying key intermediates in these processes. By strategically deleting essential *nif* genes along the biosynthetic pathways of the two clusters, a number of cluster intermediates were captured on different scaffold proteins and subsequently characterized by biochemical, spectroscopic and structural approaches. Moreover, maturation of each intermediate was achieved upon incubation with a select combination of purified components, further defining the roles of protein factors and the sequence of events at each assembly step. Such a combination of *in vivo* and *in vitro* approaches has led to the identification of a “minimum set” of *nif* gene products that are required for the formation of P- and M-clusters and a “gallery” of cluster intermediates that emerge sequentially along the biosynthetic pathways of these clusters. These results are discussed below (section 2 and section 3), which provide a framework for further investigations of the chemical/molecular details of nitrogenase cluster assembly and, hopefully, offer some useful hints for future development of synthetic strategies of nitrogenase clusters and analogs.

2. THE “EX SITU” BIOSYNTHESIS OF M-CLUSTER

Biosynthesis of the M-cluster (Figure 3) is initiated through the concerted actions of NifS and NifU, which lead to the formation of [Fe₄S₄] clusters on NifU.^{41–46} Further processing of these 4Fe fragments into a mature M-cluster occurs on a series of scaffold proteins outside the target NifDK protein (hence the term “*ex situ*” biosynthesis).^{35–39,86} It involves the formation of an 8Fe core on NifB, the maturation of the 8Fe core into an M-cluster on NifEN, and the transfer of the M-cluster to its target binding site in NifDK. NifB and NifEN are two key players in this process: the former hosts the coupling of two [Fe₄S₄] clusters into an [Fe₈S₉] cluster concomitant with the radical SAM-dependent insertion of a carbide atom (section 2.1); whereas the latter houses the conversion of the [Fe₈S₉] cluster to a mature M-cluster upon the NifH-mediated insertion of Mo and homocitrate (section 2.2). Upon completion of assembly, the M-cluster is transferred from NifEN to NifDK via direct protein-protein interaction and inserted into its binding site via a positively charged path (section 2.3).

2.1. Formation of an 8Fe core on NifB

NifB is believed to receive [Fe₄S₄] clusters from NifU, process these small FeS fragments into a large FeS core, and subsequently deliver this FeS core to NifEN for further maturation

(Figure 4A).^{35–39,86} The indispensable role of NifB in the biosynthesis of M-cluster was suggested earlier by the loss of nitrogenase activity in the crude extracts of *nifB*-deletion strains^{53–56} and confirmed later by the isolation and characterization of an M-cluster-deficient form of NifDK (designated *nifB* NifDK; also see section 2.3) from a *nifB*-deletion strain of *A. vinelandii*.^{53,87} However, the exact function of NifB in this process has long remained unknown. Sequence analysis reveals the presence of a sufficient amount of ligands in NifB, which can accommodate coordination of the entire complement of Fe atoms in the M-cluster. Additionally, there is a CxxxCxxC motif at the N terminus of NifB, which is a characteristic feature of radical *S*-adenosyl-L-methionine (SAM)-dependent enzymes.³⁹ These observations suggest that NifB may utilize radical chemistry at its SAM domain for the synthesis of a complete FeS core of M-cluster. Given the 4Fe nature of the precursors supplied by NifU, formation of such a core structure likely involves the coupling of two 4Fe units; moreover, this process may be accompanied by the insertion of the interstitial carbon, as radical SAM enzymes are known for their abilities to mobilize and insert carbon species.^{88–92}

Support for this hypothesis came from the recent characterization of a NifEN-B fusion protein, which was generated by fusing the 3' end of *nifN* to the 5' end of *nifB* and expressing the fusion protein in a *nifHDK*-deletion strain of *A. vinelandii*.⁵⁷ The NifEN-B fusion protein represents a “two-in-one” assembly unit comprising two adjacent biosynthetic apparatus along the biosynthetic pathway of the M-cluster. Consequently, it should accumulate two consecutive intermediates when the biosynthetic flow is interrupted by the absence of NifDK (the terminal receptor for M-cluster) and NifH (an essential factor for the maturation of M-cluster) (see Figure 3). Indeed, two transient cluster species can be captured on NifEN-B: one, designated the K-cluster, is a [Fe₄S₄] cluster pair; the other, designated the L-cluster, is a [Fe₈S₉] cluster (Figure 4B). Apart from the two transient cluster species, a permanent [Fe₄S₄] cluster, designated the SAM cluster, is present in NifB and associated with the SAM motif of this protein.⁶¹ The K- and SAM-clusters give rise to a composite $S = 1/2$ EPR signal at $g = 2.02, 1.95$ and 1.90 (Figure 4C, *upper*), which disappears upon the addition of SAM.⁶¹ This observation implies that the K- and SAM-clusters are located in close proximity to each other, and that the response of the latter to SAM triggers the conversion of the former to another cluster species. Consistent with this hypothesis, the disappearance of the $S = 1/2$ signal is accompanied by the appearance of an L-cluster-specific, $g = 1.94$ signal (Figure 4C, *lower*), which can be accounted for by the coupling of the two 4Fe modules of the K-cluster into an 8Fe L-cluster.⁶¹ Fe K-edge X-ray absorption spectroscopy/extended X-ray absorption fine structure (XAS/EXAFS) and crystallographic analyses show that the L-cluster is an [Fe₈S₉] cluster that closely resembles the core structure of a mature M-cluster (also see section 2.2 below).^{93–95} Fe K β X-ray emission spectroscopy (XES) analysis further demonstrates the presence of a carbide atom in the L-cluster, completing the stoichiometric definition of the L-cluster as an [Fe₈S₉C] cluster.⁹⁶ Together, these observations point to a novel synthetic route employed by NifB, one that couples/rearranges two [Fe₄S₄] clusters into an [Fe₈S₉C] cluster concomitant with the radical SAM-dependent insertion of carbide and the addition of “the 9th sulfur” (Figure 4B).

The pattern of SAM cleavage by NifEN-B provides the initial insights into the role of radical SAM chemistry in this process.⁶² HPLC and MS analyses (Figure 5A) show that SAM is cleaved into two products in the presence of NifEN-B: 5'-deoxyadenosine (5'-dAH) and S-adenosyl-homocysteine (SAH). Upon substitution of [methyl-*d*₃] SAM for unlabeled SAM, however, a mixture of deuterium-enriched and unlabeled 5'-dA (5'-dAD and 5'-dAH, respectively) can be detected along with SAH as products of SAM cleavage (Figure 5B).⁶² Interestingly, two radical SAM-dependent RNA methylases, RlmN and Cfr, display the same SAM cleavage and deuterium substitution patterns.^{91,92} The proposed mechanism for RlmN- and Cfr-catalyzed reactions involves an initial step of SN2-type methyl transfer from one equivalent of SAM (resulting in the formation of SAH) and a subsequent step of 5'-dA• formation from a second equivalent of SAM, followed by hydrogen atom abstraction from the methyl group by 5'-dA• (resulting in the formation of 5'-dAH). The parallelism between the reactions catalyzed by NifEN-B (or more specifically, its NifB entity) and the two RNA methylases suggests that NifB uses a similar mechanism for the mobilization of the methyl group of SAM; only in this case, the methyl group serves as the source of the interstitial carbide. Radiolabeling experiments provide compelling evidence for this proposal, showing that the incubation of NifEN-B with [methyl-¹⁴C] SAM leads to an accumulation of ¹⁴C label in this protein, which can be further traced to the L-cluster upon extraction (Figure 5C).⁶² The accumulation of ¹⁴C label in the L-cluster proves that the insertion of carbon indeed occurs upon the conversion of the K-cluster to an L-cluster; whereas the absence of ¹⁴C label from the polypeptides of the protein suggests a direct transfer of carbon to the cluster intermediates without going through a protein-bound carbon intermediate step.⁶²

Two mechanisms can be proposed for the reaction catalyzed by NifB, which differ in the carbon intermediates they generate.⁶² One mechanism involves the transfer of the methyl group of SAM to a sulfide atom of the K-cluster via an SN2 mechanism, and the subsequent formation of a methylene radical through the abstraction of hydrogen atom from this methyl group (Figure 5D); whereas the other involves the formation of a methyl radical via the reductive cleavage of SAM, the transfer of this radical to an iron atom of the K-cluster, and the subsequent rearrangement of this radical into a methylene radical (Figure 5E). Regardless of the type of carbon intermediate, the transfer of this intermediate to the K-cluster initiates radical chemistry for the bond rearrangement that is required for the subsequent restructuring/coupling of the 4Fe units of K-cluster into an 8Fe L-cluster. Moreover, the initial carbon intermediate must undergo additional deprotonation and/or dehydrogenation steps till a carbide atom is generated in the center of the L-cluster (Figure 5D and E). Finally, there must be a concomitant insertion of "the 9th sulfur" that completes the stoichiometry of the L-cluster (Figure 5D and E). The origin of this sulfur is unclear; however, given the documented ability of SAM to serve as a sulfur donor,^{88,97} it is reasonable to speculate that "the 9th sulfur" may come from SAM. Alternatively, the K-cluster may have an extra sulfur atom attached to one of its iron atoms in a way that is analogous to what has been reported for (*R*)-2-hydroxyisocaproyl-CoA dehydratase.⁹⁸ Currently, there are many unanswered questions regarding the formation of the 8Fe core of M-cluster, and further investigation is required to elucidate the mechanistic details of carbon- and sulfur-insertion in this process.

2.2. Maturation of the 8Fe core on NifEN

NifEN is believed to receive the L-cluster from NifB, mature the L-cluster into an M-cluster, and subsequently deliver the M-cluster to NifDK (Figure 6A). Sequence alignment reveals a significant degree of sequence homology between NifEN and NifDK, which has led to the hypothesis that NifEN contains analogous cluster sites to the P- and M-cluster sites in NifDK; only in the case of NifEN, its “M-cluster site” serves as a site for M-cluster assembly rather than substrate reduction.⁹⁹ While the P-cluster analog in NifEN was identified earlier as a [Fe₄S₄] cluster,¹⁰⁰ the M-cluster analog (designated the L-cluster) was captured only recently on NifEN proteins that were expressed either alone^{95,101} or as a fusion with NifB⁶¹ in a *nifHDK*-deletion background (also see section 2.1). Biochemical analyses show that the L-cluster is free of Mo and homocitrate,¹⁰¹ whereas Fe K-edge XAS/EXAFS analyses of both the NifEN-bound⁹⁵ and the NMF-extracted⁹⁴ forms of the L-cluster demonstrate that this cluster is nearly indistinguishable in structure from the metal-sulfur core of the M-cluster (Figure 6B and 7A). Crystallographic studies of NifEN⁹³ provide further support to the EXAFS-derived 8Fe model of the L-cluster,⁹⁵ showing that the electron density of this cluster is compatible in shape and extent with that of an [Fe₈S₉] structure. The capture of such a cluster on NifEN not only highlights the function of NifB in synthesizing a complete core structure of the M-cluster, but also suggests that the M-cluster is assembled by having an all-iron core in place before the insertion of Mo and homocitrate.

The L-cluster can be converted to a mature M-cluster while it is associated with NifEN, which defines the function of NifEN as an M-cluster maturase while establishing the L-cluster as a physiologically relevant precursor to the M-cluster (Figure 6B). This conversion can be achieved by incubating NifEN with NifH, MgATP, dithionite, molybdate (MoO₄²⁻) and homocitrate, which generates a NifEN-bound form of the M-cluster^{74,75,101,102} that can be subsequently used for the reconstitution and activation of *nifB* NifDK.^{74,75,102} The conversion of L-cluster to M-cluster on NifEN can be monitored by the disappearance of the L-cluster-specific, *g* = 1.94 EPR signal in the IDS-oxidized state (Figure 6C, *upper vs. middle*) and the concurrent appearance of a small, M-cluster-like EPR signal at *g* = 4.45, 3.96, 3.60 and 2.03 in the dithionite-reduced state (Figure 6D, *upper vs. middle*).^{75,102} Fe and Mo K-edge XAS/EXAFS analyses demonstrate that the structure of the M-cluster on NifEN is nearly identical to that of the M-cluster on NifDK (Figure 7B),^{75,102} except for a somewhat asymmetric coordination of Mo that likely originates from a different ligand environment in NifEN.^{75,102} Interestingly, biochemical analyses suggest that NifEN undergoes a conformational rearrangement upon the conversion of L-cluster to M-cluster, as only the M-cluster-bound NifEN can form a complex with NifDK.¹⁰³ Consistent with this suggestion, crystallographic analysis of the L-cluster-bound form of NifEN reveals an unusual, nearly surface-exposed location of the L-cluster.⁹³ This observation is somewhat unexpected, as the predicted “M-cluster” site in NifEN, like the M-cluster site in NifDK, should be buried within the protein. It can be proposed, however, that the surface location of the L-cluster permits easy access of Mo and homocitrate for the maturation of this cluster, which subsequently triggers a conformational change of NifEN that relocates the matured M-cluster from the surface of the protein to its binding site within the protein.^{35,36,38,93}

Given the homology between NifDK and NifEN, the conformational rearrangement of NifEN upon L-cluster maturation is likely induced by its interaction with NifH in a manner that is analogous to the interaction between NifDK and NifH during substrate turnover.^{15,16,104,105} Indeed, the function of NifH in L-cluster maturation, like the function of this protein in substrate turnover, is strictly dependent on ATP hydrolysis and redox potentials.^{74,75,102} However, the exact mode-of-action of this ATPase seems to be more complicated in this process. The NifH protein re-isolated after incubation with NifEN, MgATP, dithionite, molybdate (MoO_4^{2-}) and homocitrate is “loaded” with Mo and homocitrate (Figure 8A), and it can be used as a direct donor of these two missing components to the NifEN-bound L-cluster.⁷⁴ Mo K-edge XAS analysis (Figure 8B) suggests that the Mo species is “processed” upon binding to NifH, displaying a change in its formal oxidation state and/or ligation pattern.⁷⁴ EPR analysis reveals an interesting line-shape of the “loaded” NifH (Figure 8C, *lower*) that is intermediary between those of the ADP- and ATP-bound forms of NifH,⁷⁴ which coincides with the observation of Mo at a position corresponding to the γ -phosphate of ATP in the crystal structure of an ADP-bound form of NifH.¹⁰⁶ Biochemical analysis further indicates an obligate co-mobilization of Mo and homocitrate, showing that the L-cluster cannot be converted to an M-cluster upon stepwise incubation with Mo and homocitrate.¹⁰³ Combined results of these studies suggest a binding pattern of ADP-Mo-homocitrate at the nucleotide-binding site of the “loaded” NifH (Figure 8A, *lower*), which may represent the initial step of Mo mobilization by this protein; moreover, they define a second function of NifH as an ATP-dependent Mo/homocitrate insertase, which plays a key role in the maturation of L-cluster on NifEN.⁷⁴

2.3. Transfer of the M-cluster to NifDK

The completion of M-cluster assembly on NifEN signals the transfer of this cluster from NifEN to NifDK. Biochemical analysis suggests that the maturation of L-cluster on NifEN triggers a conformational change of this protein, which allows formation of a complex between NifEN and NifDK.¹⁰³ Subsequently, the M-cluster is delivered from NifEN to NifDK via direct protein-protein interaction (Figure 9A), as the reconstitution of *nifB* NifDK can be readily achieved upon incubation with the M-cluster-bound form of NifEN.^{75,102} Sequence analysis provides the first clue to the mechanism of such a direct transfer mechanism, showing that, despite the presence of homologous M-cluster binding sites in NifEN and NifDK, certain residues that either provide a covalent ligand to the M-cluster or tightly pack the M-cluster within the polypeptide matrix of NifDK are not duplicated in the sequence of NifEN.⁸⁶ It can be postulated, therefore, that the M-cluster binding sites in NifEN and NifDK are well aligned upon complex formation, which enables the “diffusion” of M-cluster from the transient binding site in NifEN (the “low affinity” site) to the target binding site in NifDK (the “high affinity” site).^{35,103} While the differential affinities of the M-cluster sites in NifEN and NifDK remain to be proven, the presence of these analogous sites in the two proteins has been established by comparing the crystal structure of the L-cluster-bound NifEN (designated NifEN)⁹³ with the crystal structures of both the wild-type NifDK (designated holo NifDK) and the M-cluster-deficient *nifB* NifDK (designated apo NifDK) (Figure 9B).^{11,12,87} In addition, a cluster insertion path, which extends from the protein surface to the M-cluster binding site within the protein, has been identified through the comparative structural analyses of these proteins. Such a path is

completely open in the case of apo NifDK (due to the absence of M-cluster from this protein), partially closed in the case of NifEN (upon the docking of L-cluster at the entrance of the path), and completely closed in the case of holo NifDK (following the insertion of M-cluster and the concomitant conformational rearrangement of the protein) (Figure 9C).

The cluster insertion path in apo NifDK has been shown to be rich in positively charged residues (Figure 9C, *upper*), which may facilitate the insertion of the negatively charged M-cluster through charge-charge interactions.^{35–39,86,87} The identification of an analogous insertion path in NifEN, which has the homocitrate-free L-cluster “stuck” at the entrance (Figure 9B and C, *middle*),⁹³ provides indirect support for the role of homocitrate in cluster insertion; more importantly, it suggests that, upon maturation, the M-cluster is relocated to its binding site within NifEN in a process that parallels the M-cluster insertion in NifDK.¹⁰³ Given the structural homology between NifEN and NifDK, a common pathway of cluster insertion can be proposed for the two proteins (Figure 9A), which consists of an initial apo conformation with an open insertion path (represented by apo NifDK), an intermediary conformation with the cluster docked at the entrance of the path (represented by NifEN), and the holo conformation with the cluster inserted at its binding site (represented by holo NifDK). The two pathways are connected through the protein-protein interaction between NifEN and NifDK, which facilitates the release of M-cluster from its transient binding site in NifEN back to the surface of the protein. Subsequently, the M-cluster is “deposited” onto the surface of NifDK, likely through the coordinated actions of cysteinyl ligands, such as the Cys^{α25} in NifEN and the corresponding Cys^{α45} in NifDK.^{36,38,93} Such a “relay” mechanism enables an efficient intermolecular transfer of M-cluster between the two proteins, preventing the cluster from escaping into the surrounding environment.

Once it reaches the surface of NifDK, the M-cluster interacts with a number of NifDK residues *en route* to its target binding site. These residues are located in three key regions along the insertion path in apo NifDK (Figure 10). The first region, designated the “lid loop” region, consists of a polypeptide stretch between residues α353 and α364. Among these residues, His^{α362}, which is located at the tip of the “lid loop”, could serve as a transient ligand for the M-cluster at the entrance of the insertion path. The second region, designated the “His triad” region, consists of three histidine residues: His^{α274}, His^{α442} and His^{α451}. These residues may provide an intermediary docking point for the M-cluster halfway down the insertion path. The third region, designated the “switch/lock” region, consists of two residues: His^{α442} and Trp^{α444}. These residues could secure the M-cluster at its binding site by the bulky side chain of Trp^{α444} following a switch in position with His^{α442}.⁸⁷ Mutational analyses provide support for the proposed roles of these residues, showing a specific and significant decrease in the level of M-cluster incorporation upon removal of the positive charge, the ligand capacity and the steric effect at these positions.^{107–109} The insertion of M-cluster is accompanied by a conformational rearrangement of NifDK. Small-angle X-ray scattering (SAXS) analyses demonstrate that apo NifDK ($R_g = 42.4 \text{ \AA}$) is present in a more extended conformation than holo NifDK ($R_g = 40.2 \text{ \AA}$), suggesting that the overall conformation of the protein is compacted upon M-cluster insertion.¹¹⁰ Such a conformational rearrangement “closes” the insertion path and “buries” the M-cluster at a location that is $\sim 10 \text{ \AA}$ below the surface of the protein (Figure 9B and C, *lower*). The

structure of the M-cluster receives a final “touchup” upon proper ligation at this site, completing the biosynthetic process of this unique metallocofactor.

3. THE “*IN SITU*” BIOSYNTHESIS OF P-CLUSTER

Biosynthesis of the P-cluster (Figure 11), like that of the M-cluster, is launched by NifS and NifU, which mobilize Fe and S for the generation of small $[\text{Fe}_4\text{S}_4]$ clusters.^{41–46} Further processing of these small FeS fragments into a mature P-cluster takes place within the target NifDK protein (hence the term “*in situ*” biosynthesis),^{36,38,86} and it involves the reductive coupling of paired $[\text{Fe}_4\text{S}_4]$ -like clusters (section 3.1). NifH and NifZ are two key players in this process, orchestrating the stepwise assembly of the two P-clusters in the two $\alpha\beta$ -halves of NifDK (section 3.2). The formation of P-cluster triggers conformational rearrangements of NifDK which, in turn, permits the insertion of the M-cluster into the protein (section 3.3).

3.1. Reductive coupling of paired 4Fe clusters on NifDK

The geometric symmetry of P-cluster suggests that this 8Fe cluster may be assembled through the fusion of two 4Fe subclusters. This type of reaction has been well established in synthetic chemistry¹¹¹ and successfully carried out in the chemical synthesis of P-cluster topologs from 4Fe precursors.^{112–114} The initial indication that biological systems could also utilize such a synthetic route came from early attempts to extract the P-cluster from NifDK, which resulted in the recovery of 4Fe fragments.² However, direct evidence in this regard was obtained only recently through the characterization of two NifDK variants from *A. vinelandii*. One of these variants, designated *nifB* NifDK, was isolated from a *nifB*-deletion strain; the other, designated *nifH* NifDK, was isolated from a *nifH*-deletion strain. Neither NifDK variant contains M-cluster due to the absence of NifB or NifH, two essential protein factors for M-cluster biosynthesis (also see section 2). However, *nifB* NifDK carries a “standard” P-cluster (Figure 12A–C, *lower*),⁸⁷ showing a $g = 11.8$ parallel-mode EPR signal in the IDS-oxidized state that is characteristic of the P^{OX} state (Figure 12E, *lower*);⁴⁷ in contrast, *nifH* NifDK contains an unusual “P-cluster”, showing an $S = 1/2$ EPR signal in the dithionite-reduced state that is characteristic of $[\text{Fe}_4\text{S}_4]^{1+}$ clusters (Figure 12D, *upper*).⁴⁷ Fe K-edge XAS/EXAFS analysis reveals that the “P-cluster” in *nifH* NifDK (designated the P^* -cluster) is a $[\text{Fe}_4\text{S}_4]$ -like cluster pair comprising a normal $[\text{Fe}_4\text{S}_4]$ cluster and an atypical $[\text{Fe}_4\text{S}_4]$ cluster that is either distorted (*i.e.*, by having a bridging Cys in place of a core sulfide) or coordinated by a light atom (*e.g.*, N or O from Asp, His, Ser or adventitious water) (Figure 12A–C, *upper*).^{48,49} Magnetic circular dichroism (MCD) analysis provides further support for the P^* -cluster model that consists of a pair of unbridged 4Fe subclusters, assigning one subcluster as a ferredoxin-type, $[\text{Fe}_4\text{S}_4]^{1+}$ cluster and the other a diamagnetic, $[\text{Fe}_4\text{S}_4]$ -like cluster in the dithionite-reduced state.^{115,116}

The P^* -cluster can be converted to a mature P-cluster upon incubation of *nifH* NifDK with NifH, MgATP and dithionite.⁴⁹ Such a conversion is reflected by the disappearance of the P^* -specific, $S = 1/2$ EPR signal in the dithionite-reduced protein (Figure 12D, *upper vs. lower*) and the concomitant appearance of the P^{OX} -specific, $g = 11.8$ EPR signal in the IDS-oxidized protein (Figure 12E, *upper vs. lower*).⁴⁹ Additionally, Fe K-edge XAS/EXAFS analysis reveals an elongation of the average Fe-S backscattering distance, as well as a change in the Fe-Fe backscattering components, both consistent with the conversion of the

P*-cluster in *nifH* NifDK to a mature P-cluster.⁴⁹ These spectroscopic changes can be further correlated with an increase in the reconstituted activity of the protein upon P-cluster maturation.⁴⁹ Together, these observations firmly establish the P*-cluster as a physiologically relevant precursor to the P-cluster. Interestingly, Fe K-edge XAS analysis demonstrates that the Fe atoms of the P*-cluster undergo a successive reduction during the course of maturation till they eventually stabilize at a near all-ferrous oxidation state.⁴⁹ Consistent with this observation, maturation of the P-cluster can be maximized at certain concentrations of reductant (dithionite) and reductase (NifH), and it is strictly dependent on the hydrolysis of ATP, suggesting a critical role of redox chemistry in this process.⁴⁹

The strict ATP- and redox-dependence of P-cluster assembly points to a third role of NifH, one that is involved in the reductive coupling of two 4Fe subclusters (P*-cluster) into an 8Fe cluster (P-cluster). NifH likely interacts with NifDK in a similar manner in P-cluster assembly and substrate reduction, both of which involve the complex formation between NifH and NifDK, the conformational rearrangements of the two proteins, and the electron transfer from NifH to NifDK. However, in the case of P-cluster assembly, NifH plays a role in tuning the redox potentials of the two 4Fe subclusters and placing them in a proper orientation for efficient coupling. Consistent with this hypothesis, SAXS analysis reveals a 6 Å gap at the α/β -subunit interface of *nifH* NifDK (Figure 12A, *upper*), which is absent from the structure of *nifB* NifDK (Figure 12B, *lower*).¹¹⁰ Since *nifH* NifDK and *nifB* NifDK represent the conformations of NifDK before and after the maturation of P-cluster, this observation suggests that NifH induces a conformational change that brings the α - and β -subunits of NifDK together, which then facilitates the coupling of the two [Fe₄S₄]-like subclusters in these two subunits.

Despite the progress in identifying the relevant intermediate and the plausible mechanism of P-cluster biosynthesis, the chemical details of this process are yet to be defined. In particular, it is not clear how “the 8th sulfur” is eliminated upon the coupling of two [Fe₄S₄] clusters into an [Fe₈S₇] cluster. Recently, it was reported that an [Fe₈S₇] core could be chemically synthesized from two all-ferric [Fe₄S₄] clusters via phosphine desulfurization.¹¹² By analogy, biosynthesis of the P-cluster could also involve the reductive desulfurization of one [Fe₄S₄] subcluster. Alternatively, if one of the two 4Fe subclusters is in fact ligated by three S atoms and one light atom (*e.g.*, N or O), then it could be directly fused with the other [Fe₄S₄] cluster without the need to eliminate “the 8th sulfur”. Clearly, further investigation is required to elucidate the chemical details of this process.

3.2. Stepwise biosynthesis of the two P-clusters in NifDK

The two P-clusters are synthesized one at a time in the two $\alpha\beta$ -halves of NifDK (Figure 11). The first indication of the stepwise assembly of P-clusters came from the characterization of a *nifB nifZ* NifDK variant, which was isolated from a *nifB/nifZ*-deletion strain of *A. vinelandii*. The *nifB nifZ* NifDK variant contains both P*- and P-clusters, displaying the P*-specific, $S = 1/2$ signal in the dithionite-reduced state and the P^{OX}-specific, $g = 11.8$ parallel-mode signal in the IDS-oxidized state.⁵⁰ Quantitative analysis further illustrates that the $S = 1/2$ signal of *nifB nifZ* NifDK is ~50% in intensity compared to that of *nifH* NifDK (containing two P*-clusters), and the $g = 11.8$ signal of *nifB nifZ* NifDK is ~50%

in intensity compared to that of *nifB* NifDK (containing two P-clusters).⁵⁰ Consistent with this result, MCD analysis shows a near-perfect overlay between the actual spectrum of *nifB nifZ* NifDK and a simulated, composite spectrum of “half” *nifH* NifDK and “half” *nifB* NifDK,⁵¹ whereas activity analysis demonstrates a 50% reconstituted activity of *nifB nifZ* NifDK relative to that of *nifH* NifDK.⁵⁰ Together, these observations suggest that *nifB nifZ* NifDK represents an intermediary conformation between *nifH* NifDK and *nifB* NifDK, which contains a fully assembled P-cluster in one $\alpha\beta$ -dimer and a P*-cluster-like precursor in the other $\alpha\beta$ -dimer.^{50,51} Given the content of mature P-clusters in the three NifDK variants, *nifH* NifDK, *nifB nifZ* NifDK and *nifB* NifDK may very well represent three sequential snapshots of the coordinated biosynthesis of the two P-clusters in NifDK (Figure 11).

Indeed, the conformations of *nifB nifZ* NifDK and *nifB* NifDK can be captured sequentially during the maturation process of the P-clusters on *nifH* NifDK. When *nifH* NifDK is incubated with NifH, MgATP and dithionite with increasing lengths of time, there is a gradual increase in the magnitude of the P^{OX} -specific EPR signal (Figure 13A, *blue*), which aligns well with the increase in the reconstituted activity of this protein (Figure 13B, *blue*).⁴⁹ However, the increases in both the P^{OX} -specific signal and the reconstituted activity have a “lag” phase at approximately 50% of their respective maximum values. Given the presence of two P-clusters in NifDK, the half P-cluster content at the “lag” phase marks the completion of P-cluster assembly in the “first” $\alpha\beta$ -half of the protein and the achievement of a protein conformation represented by *nifB nifZ* NifDK. With longer incubation time, *nifH* NifDK can be further matured beyond the “lag” phase, showing a doubling of the magnitude of the P^{OX} -specific signal (Figure 13A, *blue*) and a doubling of the enzymatic activity (Figure 13B, *blue*) at the end point of maturation. Interestingly, the post “lag” maturation of *nifH* NifDK aligns well with the maturation of *nifB nifZ* NifDK in terms of spectroscopic changes and enzymatic activities (Figure 13A and B, *red*),^{38,52} both resulting in the formation of P-cluster in the “second” $\alpha\beta$ -half of the protein and the achievement of a protein conformation represented by *nifB* NifDK. Fe K-edge XAS/EXAFS analysis provides further evidence for the biphasic pattern of P-cluster maturation in *nifH* NifDK, showing an unevenly paced shift of Fe K-edge energy,⁴⁹ as well as a change in the ratio of short to long Fe-Fe distance in two distinct steps (Figure 13C). Together, these observations provide dynamic proofs for the stepwise biosynthesis of the two P-clusters in NifDK, which involves the formation of one P-cluster in the “first” $\alpha\beta$ -dimer prior to the formation of the other P-cluster in the “second” $\alpha\beta$ -dimer (Figure 11).

It should be noted that the maturation of the “second” P-cluster in *nifH* NifDK only requires one protein factor, NifH;⁴⁹ whereas the maturation of the “second” P-cluster in *nifB nifZ* NifDK requires the combined action of NifZ and NifH (Figure 11).⁵² This observation is not too surprising, considering that *nifH* NifDK is expressed in a *nifZ*-intact background and, therefore, has already “seen” the action of NifZ. The exact function of NifZ, however, has remained unclear. It has been proposed that this protein may act in a chaperon-like function and modify some key residues at the “second” α/β -subunit interface that are specifically required for the coupling of the “second” $[Fe_4S_4]$ -like cluster pair.^{50,52} While this hypothesis is yet to be tested, it is clear that NifZ plays an essential role in the

assembly of the “second” P-cluster and, together with NifH, it defines a unique, unsynchronized biosynthetic mechanism of the two P-clusters in the two $\alpha\beta$ -halves of NifDK.

3.3. Coordinated assembly events upon P-cluster maturation

The biosynthesis of P-clusters in NifDK triggers a series of coordinated assembly events in this multimeric protein. Prior to the maturation of P-cluster, the M-cluster site in *nifH* NifDK assumes a “closed” conformation that does not allow the access of M-cluster (Figure 11). Upon the maturation of P-cluster and the concomitant conformational rearrangement of NifDK, however, the M-cluster site is converted to the same, “open” conformation as that of the M-cluster site in *nifB* NifDK, which subsequently allows the incorporation of M-cluster (Figure 11).⁸⁷ Thus, the assembly of NifDK occurs in a highly coordinated manner: the two P-clusters are matured one at a time in the two $\alpha\beta$ -dimers; and the insertion of M-cluster follows the maturation of P-cluster within each $\alpha\beta$ -dimer (Figure 11). The conversion of protein conformation from one that is represented by *nifH* NifDK to one that is represented by *nifB* NifDK “seals up” the α/β -subunit interface (Figure 12A and B), which in turn protects the P-cluster from solvent exposure and provides added stability to the protein structure.¹¹⁰ Moreover, such a conversion “tightens up” the protein structure, as SAX analysis reveals that *nifB* NifDK ($R_g = 42.4$ Å) exists in a less extended conformation than *nifH* NifDK ($R_g = 45.7$ Å). The structure of protein is further compacted upon M-cluster insertion (see section 2.3), completing the assembly process of this multimeric metalloprotein.

4. DISCUSSION

There is a certain parallelism between the biosyntheses of M- and P-clusters, both of which follow a route of $2\text{Fe} \rightarrow 4\text{Fe} \rightarrow 8\text{Fe}$ for the construction of a complete core structure (Figure 14). The fact that both biosynthetic pathways involve the fusion of paired 4Fe fragments into an 8Fe entity renders a topological resemblance between the two clusters, which is illustrated by the successful chemical synthesis of P- and M-cluster topologies via the rearrangement of similar core structures.^{113,114,117} Moreover, such a topological similarity implies that the $[\text{Fe}_4\text{S}_4]$ cluster may represent a common evolutionary precursor to these clusters, which is supported in part by the presence of a $[\text{Fe}_4\text{S}_4]$ cluster at the analogous position of the P-cluster in BchNB, the catalytic component of dark-operative protochlorophyllide reductase (DPOR) that is related to NifDK in evolution.^{118–120} Despite the similarity in forming a 8Fe core, the biosynthetic pathways of the P- and M-clusters diverge at/beyond this point, and the subsequent differential modifications of the 8Fe core lead to the formation of two structurally and functionally distinct clusters. The assembly of M-cluster is clearly more complicated than the assembly of P-cluster, which involves the insertion of additional elements (S and C), the rearrangement of “equatorial” Fe atoms, and the replacement of one terminal Fe atom by Mo and homocitrate. These extra processing steps may account for the more elaborate, *ex situ* synthetic route taken by the M-cluster. The *ex situ* biosynthesis is observed in the case of another complex metallocofactor, molybdopterin, providing support to this argument.³⁹

Some general themes of metallocofactor biosynthesis can be derived from the studies of nitrogenase cluster assembly. For example, the nucleotide-dependent mobilization of Mo by NifH is paralleled by the formation of an adenylylated molybdate intermediate in the biosynthesis of molybdopterin cofactors.³⁹ In addition, CooC, an analogous ATPase to NifH, has been implicated in the insertion of nickel into the C cluster of the carbon monoxide dehydrogenase (CODH),¹²¹ which likely involves the formation of a similar nucleotide-metal conjugate to that observed in the case of NifH. Thus, the mobilization of Mo by NifH may exemplify a general scheme for metal trafficking in metallocofactor assembly. As another example, the H cluster of [FeFe]-hydrogenase is synthesized in a process that involves the insertion of a [Fe₂S₂] cluster along a pathway into the assembly site within the protein and the radical SAM-dependent attachment of CO, CN⁻ and dithiolate ligands to this cluster.^{122–125} These events parallel the cluster insertion and the radical SAM-dependent carbon insertion in M-cluster assembly, which may represent a general mechanism of the transfer and modification of clusters during the assembly process of complex metallocofactors. In general, nitrogenase is an excellent model system for metalloprotein assembly, which is not only suitable for investigating the various types of chemistry involved in cluster synthesis (*e.g.*, the reductive coupling of FeS units, the elimination/addition of S, and the radical-based insertion of C), but also ideal for studying the conformational rearrangement of the host protein that accompanies and accommodates the cluster assembly (*e.g.*, the transfer/insertion of the cluster, and the stepwise assembly of the two $\alpha\beta$ -dimers). Future investigations will focus on elucidating the chemical/molecular details of these events, which will hopefully lead to a comprehensive understanding of the biosynthesis of nitrogenase metalloclusters.

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Britt Hedman received a B.S., M.S. and Ph.D. degree in Chemistry from the University of Umeå, Sweden. She was Assistant Professor at the same institute and is now Professor of Photon Science and Science Director of the Stanford Synchrotron Radiation Lightsource at Stanford University. Professor Hedman's research program is focused on the development of methodology and instrumentation for x-ray absorption spectroscopy (XAS) and application to studies of electronic and geometric structures of active sites in metalloenzymes and generally in structural biology, bioinorganic mimetic systems, and inorganic systems. These methodologies have contributed significantly to the understanding of key structural aspects of the active sites of important metalloproteins, including nitrogenase, iron-sulfur cluster-containing enzymes, blue and multi-copper proteins, and iron-containing oxidases.

Figure 1.

Crystal structure of the ADP•AlF₄⁻-stabilized NifH/NifDK complex (A) and the relative positions of components involved in the transfer of electrons (B). The two subunits of NifH are colored gray and light brown, and the α - and β -subunits of NifDK are colored red and light blue, respectively, in A. The same subunits are rendered transparent in the background in B. All clusters and ADP•AlF₄⁻ are shown as space-filling models. Atoms are colored as follows: Fe, light purple; S, gold; Mo, brown; O, red; C, dark gray; N, dark blue; Mg, green; Al, beige; F, light blue. PYMOL was used to create this figure (PDB ID: 1N2C).

Figure 2.

Crystal structures of the P^N (A) and P^{OX} (B) states of the P-cluster and the M-cluster (C). The clusters are shown as ball-and-stick models. The atoms are shown as transparent balls and colored as those in Figure 1; and the ligands are shown as sticks.

PYMOL was used to create this figure (PDB IDs: 1M1N and 3MIN). HC, homocitrate.

Figure 3.

Biosynthesis of the M-cluster. This process is launched by NifS and NifU, which mobilize Fe and S for the sequential formation of $[\text{Fe}_2\text{S}_2]$ and $[\text{Fe}_4\text{S}_4]$ clusters. Subsequently, a pair of $[\text{Fe}_4\text{S}_4]$ clusters (designated the K-cluster) are coupled into an $[\text{Fe}_8\text{S}_9]$ cluster (designated the L-cluster) on NifB concomitant with the radical SAM-dependent carbide insertion. The L-cluster is then converted to a mature M-cluster on NifEN upon insertion of Mo and homocitrate (HC) by NifH, followed by the transfer of the M-cluster to its target location in NiDK. The permanent metal clusters are colored grey and distinguished by shape, with $[\text{Fe}_2\text{S}_2]$ clusters shown as diamonds, $[\text{Fe}_4\text{S}_4]$ clusters shown as cubes, and P-clusters shown as rounded rectangles. The transient cluster intermediates are colored in a yellow/orange tone. Solid arrows connect events of cluster transformation within the same protein; whereas dashed arrows indicate cluster transfer between proteins.

Figure 4.

Schematic presentation (A), structural details (B) and the accompanying spectral changes (C) of the formation of L-cluster on NifB. (A and B) NifU supplies [Fe₄S₄] clusters to NifB, which forms an [Fe₈S₉] L-cluster through the coupling of the two [Fe₄S₄] modules of the K-cluster concomitant with the insertion of a sulfur atom and a carbon atom in a radical SAM-dependent process. The L-cluster is subsequently transferred to NifEN. The clusters and the SAM molecule are shown as ball-and-stick models, with the atoms colored as those in Figure 1. PYMOL was used to create this figure (PDB ID: 3PDI). (C) The K-cluster and the SAM-cluster collectively give rise to a SAM-responsive, $S = 1/2$ signal at $g = 2.02$, 1.95 and 1.90 in the dithionite-reduced state (*upper*); whereas the L-cluster displays a characteristic $g = 1.94$ signal in the IDS-oxidized state (*lower*).

Figure 5.

Cleavage of SAM (A), formation of 5'-dAD (B) and incorporation of ¹⁴C into the L-cluster (C) by NifEN-B, and proposed mechanisms of carbon insertion by NifB during the process of L-cluster formation (D and E). (A) The HPLC elution profile of the cleavage products of SAM (*i.e.*, SAH and 5'-dAH) upon incubation with NifEN-B and dithionite (*trace 4*). The elution profiles of SAM (*trace 1*), SAH (*trace 2*) and 5'-dAH (*trace 3*) standards are also shown in this figure. (B) LC-MS analysis showing the formation of 5'-dAD, along with 5'-dAH, upon incubation of [methyl-¹⁴C] SAM with NifEN-B. (C) Autoradiograph of the ¹⁴C-labeled L-cluster extracted from NifEN-B after incubated with [methyl-¹⁴C] SAM. (D and E) Both carbon insertion mechanisms involve hydrogen atom abstraction from a SAM-derived methyl group, radical-mediated methyl transfer to the K-cluster, and continued deprotonation till an interstitial carbide atom is formed in the center of the L-cluster. However, the initial step in D involves the transfer of methyl group via an SN2 mechanism, followed by the formation of a methylene radical upon hydrogen atom abstraction by 5'-dA• and the subsequent transfer of this radical intermediate to a S atom of the K-cluster; whereas the initial step in E involves the formation of a methyl radical via reductive cleavage of SAM, followed by the transfer of this transient intermediate to an Fe atom of the K-cluster and the subsequent processing of this intermediate into a methylene radical. The clusters are shown as ball-and-stick models, with the atoms colored as those in Figure 1. PYMOL was used to create this figure (PDB ID: 3PDI).

Figure 6.

Schematic presentation (A), structural details (B) and the accompanying spectral changes (C and D) of the maturation of M-cluster on NifEN. (A and B) NifEN hosts the conversion of L- to M-cluster upon the replacement of a terminal Fe atom by Mo and homocitrate. NifH serves as an ATP-dependent Mo/HC insertase in this process. Following the completion of M-cluster assembly on NifEN, M-cluster is delivered to its destined location in NifDK. The permanent metal clusters are colored grey, with the $[\text{Fe}_4\text{S}_4]$ clusters and the P-clusters shown as cubes and rounded rectangles, respectively. The transient cluster intermediates are colored in a yellow/orange tone. Mo, molybdenum; HC, homocitrate. Clusters are shown as ball-and-stick models, with the atoms colored as those in Figure 1. PYMOL was used to generate this figure (PDB IDs: 3PDI and 1M1N). (C and D) The L-cluster, along with the permanent $[\text{Fe}_4\text{S}_4]$ clusters on NifEN, gives rise to a composite $S = 1/2$ signal at $g = 2.09$, 1.91 and 1.85 in the dithionite-reduced state (D, upper); however, this cluster can be recognized by a unique $g = 1.94$ signal in the IDS-oxidized state (C, upper). Upon maturation, the M-cluster on NifEN displays a small signal at $g = 4.45$, 3.96 , 3.60 and 2.03 in the dithionite-reduced state (D, middle, left). The same $g = 3.96$ and $g = 2.03$ features of the M-cluster are also observed in the IDS-oxidized state (C, middle). The $S = 1/2$ signal in the dithionite-reduced state (D, middle, right) originates solely from the permanent $[\text{Fe}_4\text{S}_4]$ clusters on NifEN, as the portion of the $S = 1/2$ signal that originates from the L-cluster disappears upon the conversion of L- to M-cluster. Upon delivery to NifDK, the M-cluster exhibits an $S = 3/2$ signal at $g = 4.31$, 3.67 and 2.01 in the dithionite-reduced (D, lower), and it is EPR silent in the IDS-oxidized state (C, lower).

Figure 7.

Fe K-edge XAS spectra (A) of the L-cluster (blue) and the M-cluster (red) in the extracted state and Mo K-edge XAS spectra (B) of the M-clusters on NifEN (blue) and NifDK (red). A high degree of similarity is observed between the Fe K-edge XAS spectra of the L- and M-clusters and between the Mo K-edge XAS spectra of NifEN- and NifDK-bound M-clusters.

Figure 8.

Schematic presentation (A) and the accompanying spectral changes (B and C) of the mobilization of molybdenum and homocitrate by NifH. (A) Molybdenum (Mo) and homocitrate (HC) can be “loaded” on NifH in the presence of ATP and reductant. Mo is reduced in this process (indicated as Mo^{ox}→Mo^{red} in the figure), and it may enter NifH by attaching to the position that corresponds to the γ -phosphate of ATP upon ATP hydrolysis. Subsequently, the “loaded” NifH delivers Mo and HC to the NifEN-associated L-cluster and transforms it into a fully matured M-cluster (also see Figure 6). Mo is shown as a brown sphere; and homocitrate is shown as a ball-and-stick model. The [Fe₄S₄] cluster of NifH is represented by a gray cube. (B) Normalized Mo K-edge XAS spectra of molybdate (*upper*) and the Mo/HC-“loaded” NifH (*lower*). A reference line is drawn at 20,020 eV for the comparison of edge positions. (C) EPR spectra of the MgATP-bound NifH (*upper*) and the Mo/HC-“loaded” NifH (*lower*).

Figure 9.

Transfer of the M-cluster between NifEN and NifDK (A), and ribbon diagrams (B) and surface presentations (C) of apo NifDK, NifEN and holo NifDK. (A) NifEN and NifDK share a common cluster insertion mechanism, which involves three sequential conformations: an apo conformation containing a cluster insertion path, an intermediary conformation upon docking of the cluster at the entrance of the path, and a holo conformation following the insertion of the cluster along the path into its binding site. The biosynthetic events on NifEN and NifDK are connected via complex formation and cluster transfer between the two proteins. The permanent clusters are colored grey, with the $[\text{Fe}_4\text{S}_4]$ clusters on NifEN shown as cubes and the permanent P-clusters on NifDK shown as rounded rectangles. The transient clusters are colored in a yellow/orange tone. (B) Structures of the $\alpha\beta$ -dimers of apo NifDK (*upper*), NifEN (*middle*) and holo NifDK (*lower*), showing the location of the M-cluster sites, as well as the L- and M-clusters, in the protein. All clusters are shown as space-filling models, with the atoms colored as those in Figure 1. The permanent clusters in NifEN ($[\text{Fe}_4\text{S}_4]$ cluster) and NifDK (P-cluster) are rendered transparent in the background. (C) Electrostatic surface potentials of the $\alpha\beta$ -dimers of apo-NifDK (*upper*), NifEN (*middle*) and holo-NifDK (*lower*), showing the locations of insertion paths in these proteins. Negative surface potentials are colored red, and positive surface potentials are colored blue. PYMOL was used to prepare this figure (PDB IDs: 1L5H, 3PDI and 1M1N).

Figure 10.

Close-up of the M-cluster insertion path in apo NifDK. Some key residues for M-cluster insertion (illustrated as sticks) can be found along a positively charged insertion path that extends from the surface of the protein to the cluster-binding site within the protein. These residues include (1) the “lid loop” residue, His ^{α 362}, which may provide the first docking point for the M-cluster at the entrance of the insertion path; (2) the “His triad” residues, His ^{α 274}, His ^{α 442} and His ^{α 451}, which may provide an intermediary docking point for the M-cluster halfway down the path; and (3) the “switch/lock” residues, His ^{α 442} and Trp ^{α 444}, which may secure the FeMoco by the bulky side chain of Trp ^{α 444} through a switch in positions between the two residues at the end of the path. The electrostatic surface potentials of apo NifDK are colored as those in Figure 9.

Figure 11.

Biosynthesis of the P-cluster. This process is launched by the actions of NifS and NifU, which mobilize Fe and S for the sequential formation of $[\text{Fe}_2\text{S}_2]$ and $[\text{Fe}_4\text{S}_4]$ clusters. Subsequently, a pair of $[\text{Fe}_4\text{S}_4]$ -like clusters (designated the P*-cluster) are fused into an $[\text{Fe}_8\text{S}_7]$ P-cluster at the α/β -subunit interface of NifDK. The two P-clusters in NifDK are assembled one at a time, which renders a stepwise assembly of the two $\alpha\beta$ -dimers of this protein. The formation of the “first” P-cluster involves the sole action of NifH, whereas the formation of the “second” P-cluster requires the concerted action of NifH and NifZ. The maturation of the P-cluster not only provides added stability to each $\alpha\beta$ -subunit interface, but also induces a conformational change that “opens” up the M-cluster site, thereby allowing the insertion of the M-cluster and the completion of the assembly process of NifDK. The sequential conformations of NifDK along the biosynthetic pathway are represented by three NifDK variants: (1) *nifH* NifDK, which contains two $[\text{Fe}_4\text{S}_4]$ -like cluster pairs, one per $\alpha\beta$ -dimer; (2) *nifB nifZ* NifDK, which contains one P-cluster in one $\alpha\beta$ -dimer and one $[\text{Fe}_4\text{S}_4]$ -like cluster pair in the other $\alpha\beta$ -dimer; and (3) *nifB* NifDK, which contains two P-clusters, one per $\alpha\beta$ -dimer. The P*-cluster is shown as a pair of grey cubes at the α/β -subunit interface. All other clusters are depicted as those in Figure 3. Solid arrows connect events of cluster transformation within the same protein, whereas dashed arrows indicate cluster transfer between proteins.

Figure 12.

Structural details (A, B and C) and the accompanying spectral changes (D and E) of the coupling of a pair of [Fe₄S₄]-like clusters (designated the P*-cluster) into a mature [Fe₈S₇] P-cluster at the α/β-subunit interface of NifDK. (A and B) Ribbon diagrams of the αβ-dimers of the P*-cluster-containing *nifH* NifDK (upper) and the P-cluster-containing *nifB* NifDK (lower). The diagram on top represents the best model of *nifH* NifDK based on the SAXS data, which was constructed from the structure of holo NifDK by deletion of the M-cluster, followed by a symmetric 6 Å-translation of the α- and β-subunits about the y axis. The subunits and atoms are colored as those in Figure 1, and the polypeptides are rendered transparent in B. PYMOL was used to create this figure (PDB IDs: 1M1N and 1L5H). (C) The P*-cluster comprises one standard [Fe₄S₄] cubane (upper) and one [Fe₄S₄]-like fragment that is distorted or coordinated by additional light atoms (lower), which can be reductively coupled into a [Fe₈S₇] structure upon incubation with NifH, MgATP and dithionite. The clusters are shown as ball-and-stick models, and the atoms are colored as those in Figure 1. (D and E) EPR spectra of the cluster species of *nifH* NifDK in dithionite-reduced (D) and IDS-oxidized (E) states before (upper) and after (lower) maturation. Upon maturation, the *S* = 1/2 signal at *g* = 2.05, 1.93, and 1.90 (D, upper), which is characteristic of the P*-cluster, disappears; whereas the *g* = 11.8 parallel-mode signal (E, lower), which is characteristic of the mature P-cluster in the P^{OX} state, emerges.

Figure 13.

Time-dependent increase of the relative area of the P^{OX} -specific, $g = 11.8$ parallel-mode EPR signal (A), the relative specific activities (B) and the XAS/EXAFS-derived ratio between short and long Fe-Fe distances (C) of *nifH* NifDK (blue) and *nifB nifZ* NifDK (red). The horizontal dashed lines (A, B and C) represent the assembly of the “first” P-cluster in NifDK; whereas the vertical dashed lines (A and B) mark the starting points for the alignment of *nifH* NifDK and *nifB nifZ* NifDK data.

Figure 14.

Flow diagrams of the *"in situ"* pathway of P-cluster assembly (illustrated in the orange block) and the *"ex situ"* pathway of M-cluster assembly (illustrated in the light blue block). Biosyntheses of the P- and M-clusters share two common apparatus, NifS and NifU, at the early stages of these processes, where [Fe₄S₄] units are generated as the building blocks for the further construction of these clusters. Both pathways then use a pair of 4Fe units for the formation of an 8Fe cluster; however, this step occurs on different proteins in these pathways. In the case of P-cluster assembly, a pair of [Fe₄S₄]-like clusters (P*-cluster) are coupled into an [Fe₈S₇] cluster (P-cluster) on NifDK in a process that requires the actions of NifH and NifZ; whereas in the case of M-cluster assembly, two [Fe₄S₄] modules (K-cluster) are coupled into an [Fe₈S₉] cluster (L-cluster) on NifB concomitant with the radical SAM-dependent C insertion and the addition of S in an unknown mechanism. The L-cluster is further processed on NifEN into a [MoFe₇S₉C-homocitrate] cluster (M-cluster) upon NifH-mediated insertion of Mo and homocitrate. All clusters are shown as ball-and-stick models, with the atoms colored as those in Figure 1. PYMOL was used to create this figure (PDB IDs: 1M1N and 3PDI).

Table 1Designations and functions of the *nif* gene products.

Gene	Designation(s) of Product	Function(s) of Product
<i>nifH</i>	NifH Fe protein Component 2 Reductase component Dinitrogenase reductase Mo/homocitrate insertase	Mediates the ATP-dependent electron transfer during catalysis; facilitates the reductive coupling of a pair of 4Fe clusters into a mature P-cluster; inserts Mo and homocitrate into an 8Fe precursor of M-cluster
<i>nifD,K</i>	NifDK MoFe protein Component 1 Catalytic component Dinitrogenase	Catalyzes the reduction of substrates at its active cofactor site
<i>nifL</i>		Serves as a negative transcription regulator of <i>nif</i> genes
<i>nifA</i>		Serves as a positive transcription regulator of <i>nif</i> genes
<i>nifF</i>	NifF Flavodoxin	Involved in the transfer of electrons to nitrogenase during catalysis or assembly
<i>nifS</i>	NifS Cysteine desulfurase	Transfers sulfur to NifU for the assembly of small FeS clusters
<i>nifU</i>	NifU	Serves as a scaffold for the assembly of small FeS clusters, which can then be used for M- and P-cluster assembly
<i>nifB</i>	NifB	Mediates the radical SAM-dependent insertion of carbon concomitant with the formation of a Mo/homocitrate-free precursor of M-cluster
<i>nifE,N</i>	NifEN	Serves as a scaffold for the maturation of M-cluster; a structural/functional homolog of the MoFe protein
<i>nifV</i>	NifV Homocitrate synthase	Synthesizes homocitrate for M-cluster assembly
<i>nifZ</i>	NifZ	Serves as a key factor in the stepwise maturation of P-clusters in MoFe protein, possibly through a chaperone-like function
<i>nifM</i>	NifM Peptidyl-prolyl <i>cis-trans</i> isomerase	Involved in the maturation of Fe protein
<i>nifX</i>	NifX	Proposed intermediate carrier in M-cluster assembly
<i>nifY</i>	NifY	Proposed intermediate carrier in M-cluster assembly
<i>nafY</i>	NafY	Proposed intermediate carrier in M-cluster assembly
<i>nifW</i>	NifW	Putative role in protecting nitrogenase from oxygen damage
<i>nifO</i>	NifO	Function unknown, though it resembles thioredoxin
<i>nifT</i>	NifT	Function unknown