Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase*

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Running title: Assembly of Nitrogenase Iron-Molybdenum Cofactor

The iron-molybdenum cofactor, or the M-cluster, serves as the active site of molybdenum nitrogenase. Arguably one of the most complex metal cofactors in biological systems, the M-cluster is assembled through the formation of an 8Fe core prior to the insertion of Mo and homocitrate into this core. Here, we review the recent progress in the research area of M-cluster assembly, with an emphasis on our work that provides useful insights into the mechanistic details of this process.

Nitrogenase catalyzes the reduction of nitrogen (N₂) under ambient conditions (1-3). The molybdenum (Mo)-dependent nitrogenase consists of a reductase component (NifH) and a catalytic component (NifDK). The active site of Mo nitrogenase is called the iron-molybdenum (FeMo) cofactor, or the M-cluster (Fig. S1A). Located within NifDK (an α₂β₂-tetramer), the M-cluster receives electrons from NifH (a γ₂-dimer) in an ATP₂-assisted process and subsequently serves as the site for substrate reduction upon accumulation of sufficient electrons (Fig. S1B) (4). Arguably one of the most complex metal cofactors found in nature, the M-cluster can be viewed as [Fe₄S₃] and [MoFe₃S₃] subclusters bridged by three sulfide atoms (5, 6). Additionally, it has a homocitrate moiety attached to its Mo end, as well as a carbide atom coordinated in the center of its structure (6-8). The M-cluster is ligated to the α-subunit of NifDK by Cys⁴₂⁷⁵ at its Fe end and Hisα₄₄₂ at its Mo end, with Lysα₄₂⁶ providing an additional anchor for its homocitrate entity (5, 6).

The structural complexity and biological importance of M-cluster have prompted vigorous research on the assembly mechanism of this metal cofactor, as knowledge in this regard is not only important for understanding the structure-function relationship of nitrogenase, but also instrumental in developing future synthetic strategies for nitrogenase-based, biomimetic catalysts. Previous studies have established that the M-cluster is synthesized stepwise on a number of scaffold proteins before it is delivered to its destined location in NifDK, and that the minimum set of factors required for this process include the gene products of nifS, nifU, nifB, nifE, nifN and nifH (9). Here, we briefly review the recent progress in this research area, highlighting our work on the Mo nitrogenase from Azotobacter vinelandii that provides meaningful insights into the biosynthetic pathway of its M-cluster. An alternative view of some aspects of M-cluster assembly can be found elsewhere (10).

Synthesis of a 4Fe Cluster Pair

Biosynthesis of the M-cluster is launched by NifS and NifU, which mobilize Fe and S for the generation of small FeS fragments (Fig. 1). It is believed that NifS, a pyridoxal phosphate-dependent cysteine desulfurase, forms a protein-bound cysteine persulfide that is subsequently donated to NifU for the sequential formation of [Fe₂S₂] and [Fe₄S₄] units (11). The [Fe₄S₄] clusters are then delivered from NifU to NifB and further processed into a large FeS core (Fig. 1). This hypothesis is supported by the identification of a pair of [Fe₄S₄] clusters (designated K-cluster) on NifB, which can be easily accommodated by a sufficient amount of ligands in NifB (9). Metal analysis further reveals the presence of additional [Fe₂S₄] clusters on NifB, which are associated with the conserved CxxxCxxC motifs—a characteristic feature of radical SAM enzymes—in this protein (9). Collectively, the K-cluster and the SAM motif-associated cluster (designated the SAM-cluster) give rise to an S = 1/2 EPR signal at g = 2.02, 1.95 and 1.90 (12). More excitingly, this composite S = 1/2 signal disappears upon addition of SAM, suggesting that the K-cluster and the SAM-cluster are located in close proximity to each other (12). Such a cluster arrangement is important, as it facilitates the

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subsequent coupling of the two 4Fe units of K-clusters via a radical SAM-dependent route (see below).

**Formation of an 8Fe Core**

The K-cluster on NifB can be converted to an 8Fe core in the presence of SAM (Fig. 2A). The concomitant disappearance of the K-cluster- and SAM-cluster-originated S = 1/2 signal upon addition of SAM is accompanied by the appearance of a unique g = 1.94 signal, which has been attributed to an [Fe₈S₉] cluster (designated the L-cluster) (12). Fe K-edge XAS/EXAFS and crystallographic studies show that the L-cluster closely resembles the core structure of the mature M-cluster, except that the Mo and homocitrate components at one end of the cluster are replaced by an Fe atom (13-16). Fe Kβ XES analysis further demonstrates the presence of a carbide atom in the center of the L-cluster (17). Together, these observations imply that the insertion of carbide may occur simultaneously with the transformation of a K-cluster into an L-cluster. A novel synthetic route to the 8Fe L-cluster can be postulated, one that inserts the carbon atom via the radical chemistry at the SAM domain of NifB while attaching an additional sulfur atom and coupling/rearranging the two 4Fe units of K-cluster into an [Fe₈S₉] L-cluster at the same time (Fig. 2B).

The cleavage pattern of SAM provides the initial insights into the role of radical SAM chemistry in this process (18). In the presence of NifB, SAM is cleaved into two products: 5'-deoxyadenosine (5'-dA) and S-adenosyl-L-homocysteine (SAH). Upon substitution of [methyl-3H]-SAM for unlabeled SAM, however, a mixture of deuterium-enriched and unlabeled 5'-dA can be detected along with SAH as products of SAM cleavage (18). Interestingly, two radical SAM RNA methylases, RlmN and Cfr, display the same SAM cleavage and deuterium substitution patterns as those of NifB, which have been accounted for by an SN2 mechanism, followed by the formation of a methylene radical via the reductive cleavage of SAM, abstraction of hydrogen atom from this methyl group (Fig. 2C, left); the other involves the formation of a methyl radical via the reductive cleavage of SAM, followed by the transfer of this radical to an iron atom of K-cluster and the subsequent rearrangement of this radical into a methylene radical (Fig. 2C, right). In either scenario, the transfer of carbon intermediate to the K-cluster may play a pivotal role in coupling the two 4Fe units of K-cluster into an 8Fe L-cluster, initiating radical chemistry for the initial bond rearrangement that is required for the subsequent restructuring of cluster units. Regardless of the type of the initial carbon intermediate, it must undergo additional deprotonation and/or dehydrogenation steps till a carbide atom is generated in the center of the L-cluster (Fig. 2C).

**Maturation of the 8Fe Core**

Once formed, the L-cluster is transferred from NifB to NifEN, where it is matured into an M-cluster upon the insertion of Mo and homocitrate (Fig. 3A). Such a conversion can be achieved *in vitro* by incubating the L-cluster-bound NifEN with NifH, MgATP, dithionite, molybdate (MoO₄²⁻) and homocitrate, which generates an NifEN-bound M-cluster (Fig. 3B) that can be used for the subsequent reconstitution and activation of apo NifDK (21, 22). Maturation of the cluster species on NifEN is reflected by the disappearance of the L-cluster-specific, g = 1.94 signal and the concurrent appearance of a small, M-cluster-like signal at g = 4.45, 3.96, 3.60 and 2.03 (21, 22). Fe and Mo K-edge XAS/EXAFS analyses further confirm that the structure of the M-cluster on NifEN (Fig. 3B, right) is nearly identical to that of the M-cluster on NifDK (Fig. S1A), except for a somewhat asymmetric coordination of Mo that arises from a different ligand environment in NifEN (21, 22).

Interestingly, biochemical experiments suggest that NifEN undergoes a conformational change upon the conversion of L-cluster to M-cluster, as only the M-cluster-bound form of NifEN is capable of complex formation with NifDK (23). 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 (16). This
observation is unexpected, as the highly homologous NifEN and NifDK proteins should have homologous cluster-binding sites that are buried within their respective polypeptides. It is conceivable that the surface location of L-cluster permits an easier access of Mo and homocitrate for cluster maturation, whereas a concurrent conformational change of NifEN enables subsequent relocation of the matured cluster from the surface to the binding site within the protein (Fig. 3A).

The conformational change of NifEN is likely induced by its interaction with NifH upon cluster turnover, which parallels the interaction between the NifDK and NifH upon substrate turnover. Indeed, NifH exhibits a strict dependence on ATP hydrolysis and redox potential to carry out its function both in catalysis (1-3) and in assembly (21, 22). However, the role of this ATPase in assembly appears more complicated, as NifH can be “loaded” with Mo and homocitrate and subsequently used as a donor of these two missing components to the NifEN-bound L-cluster (24). Biochemical analysis shows a mandatory co-mobilization of Mo and homocitrate by NifH; whereas Mo K-edge XAS analysis reveals that the Mo species is “processed” upon binding to NifH, displaying a change in its formal oxidation state and/or the ligation pattern (24). More interestingly, EPR analysis suggests that NifH binds nucleotide along with Mo and homocitrate, showing that the loaded NifH gives rise to a signal that has an intermediary line-shape between those of the ADP- and ATP-bound forms of NifH (24). This observation coincides with the outcome of the initial crystallographic analysis of an ADP-bound form of NifH, which places Mo at a position that corresponds to the γ-phosphate of ATP (25). A binding pattern of NifH/ADP-Mo-homocitrate can be proposed based on these results, which could represent the initial step of Mo mobilization by NifH (Fig. 3C). Such a binding pattern parallels the formation of a possible adenylated molybdate intermediate during the biosynthesis of molybdenopterin cofactors (9). Moreover, another ATPase, CooC, has been implicated in the insertion of nickel into the C cluster of the carbon monoxide dehydrogenase from Rhodospirillum rubrum (26). Thus, the mobilization of Mo by NifH may exemplify a general scheme for metal trafficking in biological systems.

**Conclusion**

The M-cluster is assembled on a number of scaffold proteins prior to its delivery to its target location in NifDK. This process involves two key steps: the stepwise formation of an 8Fe core and the subsequent maturation of this core upon incorporation of Mo and homocitrate. The former utilizes radical SAM chemistry for the insertion of interstitial carbide, which likely occurs concomitantly with the coupling and restructuring of two 4Fe units; whereas the latter employs a nucleotide-binding protein for the mobilization of Mo and homocitrate, which may represent a common strategy for metal trafficking. Future studies will focus on the mechanistic investigation of radical SAM-dependent carbon insertion, as well as the structural details of the NifH/ATPase-based Mo mobilization.
References

FIGURE LEGENDS

FIGURE 1. **Synthesis of a 4Fe cluster pair.** *A*, NifS and NifU mobilize Fe and S for the sequential formation of [Fe$_2$S$_2$] and [Fe$_4$S$_4$] clusters, followed by the transfer of a [Fe$_4$S$_4$] cluster pair (K-cluster) from NifU to NifB for further processing. The permanent [Fe$_2$S$_2$] clusters on NifU and the SAM motif-associated [Fe$_4$S$_4$] clusters on NifB are depicted as diamonds and cubes, respectively; whereas the transient clusters on these proteins are represented by pink ovals and labeled accordingly. *B*, structural details of the [Fe$_2$S$_2$] cluster (*left*), the [Fe$_4$S$_4$] cluster (*middle*) and the [Fe$_4$S$_4$] cluster pair (*right*). The clusters are shown as ball-and-stick models, with the atoms colored as follows: Fe, orange; S, yellow. PYMOL was used to create the figure (PDB ID: 1N2C).

FIGURE 2. **Formation of an 8Fe core.** *A*, NifB catalyzes the SAM-dependent conversion of a [Fe$_4$S$_4$] cluster pair (K-cluster) to an [Fe$_8$S$_9$] cluster (L-cluster), which is subsequently delivered to NifEN. The SAM motif-associated [Fe$_4$S$_4$] clusters on NifB are depicted as cubes; whereas the transient clusters on this protein are represented by pink ovals and labeled accordingly. *B*, structural details of the K-cluster (*left*) and the L-cluster (*right*) on NifB. The clusters are shown as ball-and-stick models, with the atoms colored as follows: Fe, orange; S, yellow; O, red; C, gray; N, dark blue. PYMOL was used to create the figure (PDB ID: 3PDI). *C*, two proposed mechanisms of carbon insertion by NifB. One 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 (*left*); whereas the other 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 (*right*).

FIGURE 3. **Maturation of the 8Fe core.** *A*, NifEN houses the conversion of L-cluster to M-cluster upon NifH-mediated insertion of Mo and homocitrate. The maturation of L-cluster is likely accompanied by a transfer of the cluster from the surface of NifEN to the M-cluster binding site within the protein. The permanent [Fe$_4$S$_4$] clusters in NifEN are represented by cubes; whereas the transient clusters on this protein are represented by pink ovals and labeled accordingly. HC, homocitrate. *B*, structural details of the L-cluster (*left*) and the M-cluster (*right*) on NifEN, with one terminal Fe atom of the L-cluster replaced by Mo and homocitrate in the M-cluster through an ATP-dependent process. *C*, NifH serves as an ATP-dependent Mo/homocitrate insertase and possibly mobilizes these two missing elements in the form of NifH/ADP-Mo-homocitrate for the maturation of L-cluster. The clusters are shown as ball-and-stick models, with the atoms colored as follows: Fe, orange; S, yellow; Mo, cyan; O, red; C, gray. PYMOL was used to create the figure (PDB IDs: 3PDI and 1M1N).

FIGURE 4. **Delivery of the M-cluster to its target location.** *A*, NifEN docks on apo NifDK upon the completion of M-cluster assembly, allowing the cluster to “diffuse” from the “low affinity site” in NifEN to the “high affinity site” in NifDK. The permanent P-cluster ([Fe$_8$S$_7$]) in NifDK is represented by a pair of cubes. *B*, the conformations of NifDK before (“Apo”, *left*), during (“Intermediate”, *middle*) and after (“Holo”, *right*) the insertion of M-cluster. The “Apo” and “Holo” conformations were taken from the crystal structures of apo and holo NifDK, respectively; whereas the “Intermediate” conformation was adapted from the crystal structure of the L-cluster-bound form of NifEN. The “Intermediate” conformation has the M-cluster attached at the surface of NifDK in an analogous manner to the attachment of the L-cluster at the surface of NifEN. The apo NifDK contains an open cluster insertion funnel (*left*), which is believed to be partially closed upon the docking of M-cluster at its entrance (*middle*) and fully closed upon the incorporation of M-cluster and the accompanying structural rearrangement of NifDK (*right*). The proteins are shown as ribbon diagrams, with the α-subunits colored red and presented in the foreground and the β-subunits colored blue and rendered transparent in the background. The clusters are shown as ball-and-stick models, with the atoms colored as follows: Fe, orange; S, yellow; Mo, cyan; O, red; C, gray. PYMOL was used to create the figure (PDB IDs: 1L5H, 3PDI and 1M1N).