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Maturation of nitrogenase cofactor—the role of a class E radical SAM methyltransferase NifB

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Abstract

Nitrogenase catalyzes the important reactions of N_2-, CO- and CO_2-reduction at its active cofactor site. Designated the M-cluster, this complex metallocofactor is assembled through the generation of a characteristic 8Fe-core prior to the insertion of Mo and homocitrate that completes the stoichiometry of the M-cluster. NifB catalyzes the critical step of radical SAM-dependent carbide insertion that occurs concomitant with the insertion a "9^{th}" sulfur and the rearrangement/coupling of two 4Fe-clusters into a complete 8Fe-core of the M-cluster. Further categorization of a family of NifB proteins as a new class of radical SAM methyltransferases suggests a general function of these proteins in complex metallocofactor assembly and provides a new platform for unveiling unprecedented chemical reactions catalyzed by biological systems.

Catalysis by nitrogenase, particularly the reduction of nitrogen (N_2) to ammonia (NH_3) [1–3] and the reduction of carbon monoxide (CO) or carbon dioxide (CO_2) to hydrocarbons under ambient conditions [4–7], bears tremendous significance to environment- and energy-related areas. The "conventional" Mo-nitrogenase utilizes a specific reductase (NifH) to deliver electrons to the active cofactor site (designated the M-cluster) of the catalytic component (NifDK), where substrate reduction takes place [1]. Arguably the most complex metallcofactor utilized by biological systems, the M-cluster consists of a [$MoFe_7S_9C$] core that can be viewed as [$MoFe_3S_3$] and [Fe_4S_3] subclusters bridged by three μ_2-sulfides and one μ_6-carbide (C^{4-}) ion, and it is further coordinated by a homocitrate moiety at its Mo end [8–10]. Understanding the assembly mechanism of this complex metallocenter is not only important for elucidation of the structural-functional relationship of nitrogenase, but also crucial for future development of strategies to synthesize biomimetic complexes for production of valuable products.

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The M-cluster: a multifaceted model for metallocofactor assembly

Biosynthesis of the M-cluster is launched by the concerted action of NifS and NifU, with NifS acting as a pyridoxal phosphate-dependent cysteine desulfurase, forming a protein-bound cysteine persulfide that is donated to NifU for the sequential formation of $[Fe_2S_2]$ and $[Fe_4S_4]$ clusters (Figure 1, I) [11–14]. Subsequently, a $[Fe_4S_4]$ cluster pair (designated the K-cluster) is transferred from NifU to NifB and processed into a $[Fe_8S_9C]$ cluster (designated the L-cluster), which is nearly indistinguishable from the M-cluster in structure except for the substitution of Fe for Mo/homocitrate at one end of the cluster (Figure 1, II) [15–19]. The L-cluster is then transferred from NifB to NifEN, where it is matured into an M-cluster upon NifH-mediated insertion of molybdenum and homocitrate prior to the delivery of the M-cluster to its target location in NifDK (Figure 1, III) [20–23].

Identification of the assembly pathway of the M-cluster was facilitated by strategic deletions of essential *nif* genes [24,25], which permitted the capture of biosynthetic intermediates of the M-cluster on various *nif*-encoded (Nif) assembly proteins (*see* Figure 1). Subsequent biochemical and EPR analyses demonstrated the sequential K→L→M cluster conversion, which could be monitored by the corresponding changes of the characteristic EPR features of these cluster species in this process (Figure 1, *a*–*c*) [15,20,22–24]; whereas XAS/EXAFS and crystallographic studies provided structural proofs for the identities of these biosynthetic intermediates, showing the formation of a complete 8Fe-core of the M-cluster (*i.e.*, the L-cluster) prior to the insertion of Mo and homocitrate (Figure 1, II) [16–18] while assigning a previously-unidentified function to NifH (the reductase component of Mo-nitrogenase) as a Mo/homocitrate insertase that transforms the L-cluster into a fully-matured M-cluster (Figure 1, III) [21].

Overall, assembly of the M-cluster utilizes a fusion strategy that stepwise generates the 2Fe-, 4Fe- and 8Fe-cores. However, there are a number of variations on this theme, including the insertion of carbon and sulfur that occurs concomitant with the coupling and rearrangement of two 4Fe units of the K-cluster into an 8Fe L-cluster (*see* Figure 1, II), as well as the replacement of a terminal Fe atom of the L-cluster by Mo and homocitrate that leads to the formation of the M-cluster (*see* Figure 1, III). These variations not only render the M-cluster chemically unprecedented and functionally unique, but also establish this cluster as a multifaceted model system for the investigation of various types of chemistry that are employed for complex metallocofactor assembly. Among them, the NifB-catalyzed carbide insertion is of particular interest [26–29], as it represents a novel, radical SAM-dependent biosynthetic route to complex, bridged metalloclusters.

The NifB protein: a “radical SAM assemblase (RSA)”

NifB carries a signature CxxxCxxC motif at its N-terminus for the coordination of an *S*-adenosyl-L-methionine (SAM)-binding $[Fe_4S_4]$ cluster (designated the SAM-cluster), as well as additional ligands for the accommodation of the two $[Fe_4S_4]$ units of the K-cluster (a biosynthetic precursor to the M-cluster) (Figure 2) [24,29]. This observation has led to the hypothesis that NifB utilizes radical SAM chemistry for the coupling of the two 4Fe units of the K-cluster into an 8Fe L-cluster. EPR analysis provided the initial proof for this

hypothesis, showing the disappearance of the K-cluster-specific, $S = 1/2$ signal concomitant with the appearance of the L-cluster-specific, $g = 1.94$ signal (*see* Figure 1, *a, b*) [15] upon incubation of NifB with SAM. Subsequent HPLC analysis revealed the identities of the products of SAM cleavage by NifB as those of *S*-adenosyl-L-homocysteine (SAH) and 5'-deoxyadenosine (5'-dAH) (Figure 2, *a*) [24], suggesting the utilization of two molecules of SAM in the reaction catalyzed by NifB: one serves as the donor of a methyl group (giving rise to SAH upon removal of the methyl group); the other generates a 5'-dA• radical for hydrogen abstraction (giving rise to 5'-dAH). Isotope labeling experiments further traced the ^{14}C label to the L-cluster when NifB was incubated with [methyl-^{14}C] SAM [24,30] while demonstrating the formation of deuterated 5'-deoxyadenosine (5'-dAD) when NifB was incubated with [methyl-d_3] SAM (Figure 2, *b*) [24], thereby establishing the methyl group of SAM as the source of the interstitial carbide that undergoes an initial processing step upon hydrogen abstraction by a 5'-dA• radical (*see* Figure 2) [24].

A recent study further refined these early steps along the carbide insertion pathway. Incubation of NifB with unlabeled SAM and [methyl-d_3] SAM, respectively, resulted in the formation of methanethiol (CH_3SH) and methane-d_3-thiol (CD_3SH), respectively, upon acid quenching, suggesting transfer of the SAM-derived methyl group to the acid-labile sulfur atom of an FeS cluster (Figure 2, *c, d*) [28]. Substitution of the Fe/Se-reconstituted NifB for the Fe/S-associated NifB in the same reaction led to the formation of methylselenol (CH_3SeH), reaffirming the sulfide atom of the FeS precursor on NifB (*i.e.*, the K-cluster) as the point of methyl group attachment (Figure 2, *e*) [28]. When ally SAM—a SAM analog containing an allyl group (-CH-CH=CH_2) in place of the methyl group (-CH_3)—was incubated with NifB, SAH was detected as the sole product of SAM cleavage [28]. Interestingly, acid quenching of this incubation mixture resulted in the formation of allylthiol (CH_2=CH-CH-SH), suggesting the occurrence of allytransfer in the absence hydrogen abstraction by a 5'-dA• radical (Figure 2, *f*) [28]. This observation provides compelling evidence that methyltransfer occurs via an S_N2-type mechanism prior to the abstraction of hydrogen from this group, resulting in a carbon intermediate (*e.g.*, a methylene radical) that can be further processed into a carbide ion

While the early events of the carbide insertion process have been elucidated through these studies, the post-hydrogen-abstraction events are yet to be explored to address such questions as (*i*) how is the methyl-derived carbon intermediate processed into a carbide ion; and (*ii*) where does the "9th" sulfur originate from and how is it incorporated to complete the stoichiometry of the L-cluster? With regard to the former, it is tempting to speculate that a continuation of hydrogen abstraction from the carbon intermediate by SAM-derived 5'-dA• radicals will eventually give rise to a carbide ion, although a reaction mechanism based on acid/base chemistry could be used to accomplish the same task. With regard to the latter, one appealing scenario is that the "9th" sulfur is a "dangling sulfur" attached externally to one Fe atom of the K-cluster in an analogous manner to those observed in the cases of other radical SAM-dependent enzymes like RimO, MiaB and HydG [31–33], as well as the radical enzyme (*R*)-2-hydroxyisocaproyl-CoA dehydratase [34]; on the other hand, a protein origin of this sulfur atom cannot be excluded, particularly when drawing analogy to the recent observation that one "belt sulfur" of the M-cluster might be temporarily "parked" on a protein residue during the process of substrate turnover by nitrogenase [35–38]. Perhaps the

most important question, however, is how radical chemistry enables the rearrangement and coupling of the two 4Fe modules of the K-cluster into an 8Fe L-cluster concomitant with the insertion of both the interstitial carbide and the "9th" sulfur. While the answer to this question remains elusive as of the moment, the capability of NifB to carry out this interesting chemistry establishes this enzyme as an efficient "radical SAM assemblase (RSA)" that specializes in complex metallocofactor assembly.

The NifB family: a new class of radical SAM methyltransferases (RSMTs)

The vast majority of our current knowledge of the NifB protein was derived from studies of this protein from a soil bacterium, *Azotobacter vinelandii* (designated NifB^{Av}). Recently, two naturally "truncated" NifB homologs have been identified in two nitrogen-fixing methanogenic microorganisms: one (designated NifB^{Ma}) from the mesophilic *Methanosarcina acetivorans*; and the other (designated NifB^{Mt}) from the thermophilic *Methanobacterium thermoautotrophicum*. Unlike NifB^{Av}, NifB^{Ma} and NifB^{Mt} lack the "NifX domain"—a sequence sharing homology with NifX, an accessory protein of unclear function in nitrogenase assembly–toward the C-termini of their primary sequences [29]. However, both proteins contain the CxxxCxxC motif for the coordination of the SAM-cluster, as well as a sufficient number of conserved Cys and His residues for the accommodation of a K-cluster. Biochemical analyses further demonstrated the functional exchangeability between NifB^{Ma}/NifB^{Mt} and NifB^{Av}, showing the ability of the truncated NifB proteins to catalyze the same radical SAM-dependent carbide insertion that is coupled to the conversion of K- to L-cluster as their full-length counterpart [29]. This observation is important, as it establishes the minimum sequence requirement for a functional NifB protein that could facilitate mechanistic investigation of this protein in M-cluster assembly.

Subsequent BLAST search have led to the identification of a large number of proteins with high sequence homology to the three NifB proteins. Overall, this family of proteins can be divided into two categories—full-length and truncated–based on the presence or absence of the NifX domain toward the C-termini of their sequences (Figure 3). The hosts of most full-length NifB proteins are proteobacteria; whereas the hosts of the truncated NifB proteins are much more widespread across the microbial biorealm, ranging from euryarcheotes to firmicutes (Figure 3). Interestingly, many hosts of the truncated NifB proteins are not nitrogen-fixing organisms, suggesting that the NifB proteins in these organisms carry out other functions that are yet to be established [29].

Identification of a family of NifB proteins has led to the categorization of a new class (class E) of radical SAM methyltransferases (RSMTs) (Figure. 4) [29], a large subset of radical SAM enzymes that catalyze methylation reactions using SAM or other methyl donor molecules as co-substrates. Class E RSMTs clearly differ from the previously-defined classes B, C and D of RSMTs, which carry a cobalamin-binding domain, a HemN domain and a methylenetetrahydrofolate domain, respectively, in addition to the canonical radical SAM domain (Figure 4) [39–42]. Moreover, while sharing the same byproducts of SAM cleavage (*i.e.*, SAH and 5'-dAH), class E RSMTs do not possess a pair of conserved Cys residues in the sequences of class A RSMTs (including one that serves as the site of intermediary methylation) and, consequently, do not route methyltransfer via a protein

residue (as exemplified by NifB^{Av}, NifB^{Ma} and NifB^{Mt}) (Figure 4) [39–42]. Instead, this new class of RSMTs contain a number of conserved Cys and His residues that flank the radical SAM domain, which could potentially serve as FeS cluster-binding domains for radical SAM-based assembly of complex metallocenters (Figure 4) [29].

Concluding remarks

NifB plays a pivotal role in the maturation of nitrogenase cofactor, catalyzing radical SAM-dependent carbide insertion concomitant with the generation of a characteristic [Fe_8S_9C] core that is nearly indistinguishable from, and hence easily convertible into, the [$MoFe_7C$] core of a mature M-cluster. Classification of a family of NifB proteins as a distinct subset of RSMTs not only suggests a broader function of these proteins in the assembly of complex metallocofactors, but also opens up new avenues to study the structure and mechanism of this important protein family, both of which remain relatively uncharacterized and promise to reveal unprecedented chemical reactions catalyzed by biological systems.

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Highlights

- The carbide of nitrogense M-cluster originates from the methyl group of SAM.
- The insertion of carbide is catalyzed by NifB via a radical SAM-dependent mechanism.
- The methyl group is transferred from SAM to an sulfur atom of the M-cluster precursor.
- The transfer of methyl to the M-cluster precursor occurs before hydrogen abstraction.
- The NifB protein family represents as a new class of radical SAM methyltransferases.

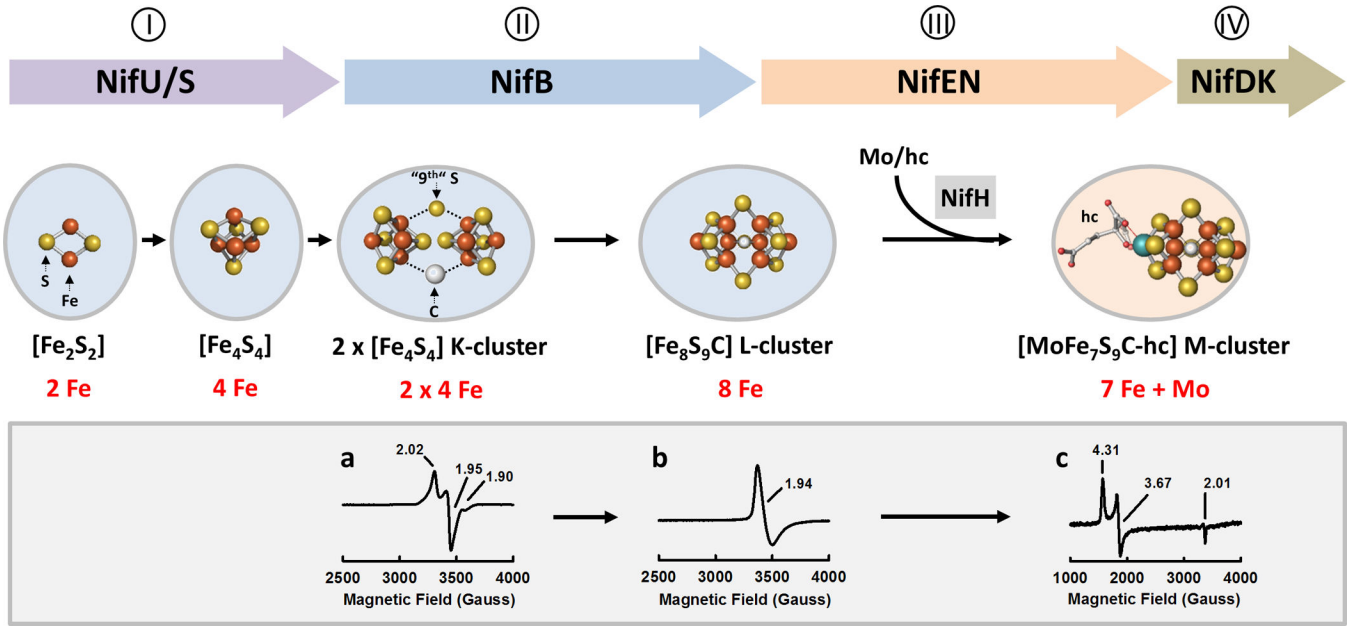

Figure 1. Assembly of nitrogenase cofactor

Actions of a series of assembly proteins lead to the sequential formation of 2Fe (on NifS/U; I), 4Fe (on NifS/U; I) and 8Fe (on NifB; II) clusters prior to the insertion of Mo and homocitrate (hc) by NifH that gives rise to a mature M-cluster (on NifEN, III) and the subsequent delivery of the M-cluster to its target location (in NifDK, IV). NifB catalyzes the K- to L-cluster conversion, which involves radical SAM-dependent carbide insertion concomitant with the insertion of a "9th" sulfur and the rearrangement/coupling of the two 4Fe units of the K-cluster into an 8Fe L-cluster. The characteristic EPR features of the (*a*) K-, (*b*) L- and (*c*) M-clusters are shown below the structural models.

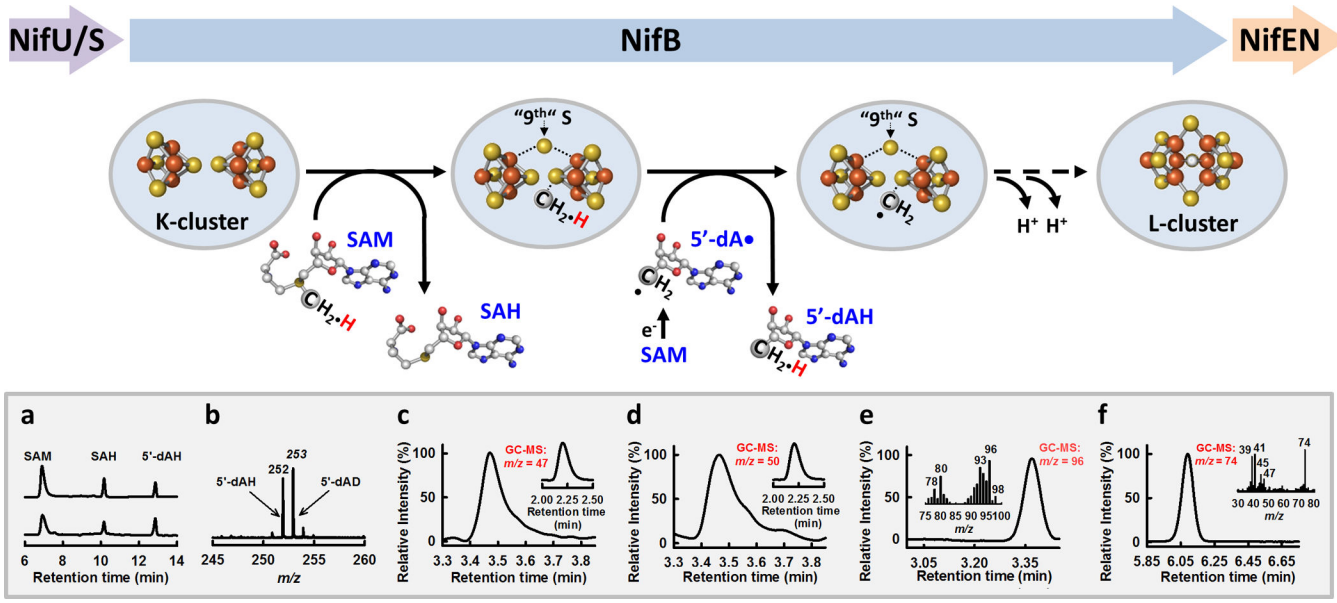

Figure 2. Formation of an 8Fe-core by NifB

Proposed pathway of carbide insertion, which begins with methyltransfer from one SAM molecule to a sulfide atom of the K-cluster, followed by hydrogen abstraction from this methyl group by a 5'-dA• radical that is derived from a second SAM molecule. The resulting, cluster-bound carbon intermediate (*e.g.*, a methylene radical) then initiates radical chemistry-based rearrangement/coupling of the two 4Fe units of the K-cluster into an 8Fe L-cluster concomitant with the insertion of a "9th" sulfur and further dehydrogenation/deprotonation of the carbon intermediate until a carbide ion appears in the center of the L-cluster. (*a*) HPLC profile of the standards (*upper*) and the actual reaction of SAM cleavage by NifB (*lower*); (*b*) LC-MS analysis of the 5'-dA species generated upon incubation of NifB with [methyl-d_3] SAM; (*c*–*f*) GC-MS analyses of products generated upon acid quenching of reactions containing (*d*) NifB and SAM; (*d*) NifB and [methyl-^{14}C] SAM; (*e*) Fe/Se-reconstituted NifB and SAM; and (*f*) NifB and ally SAM.

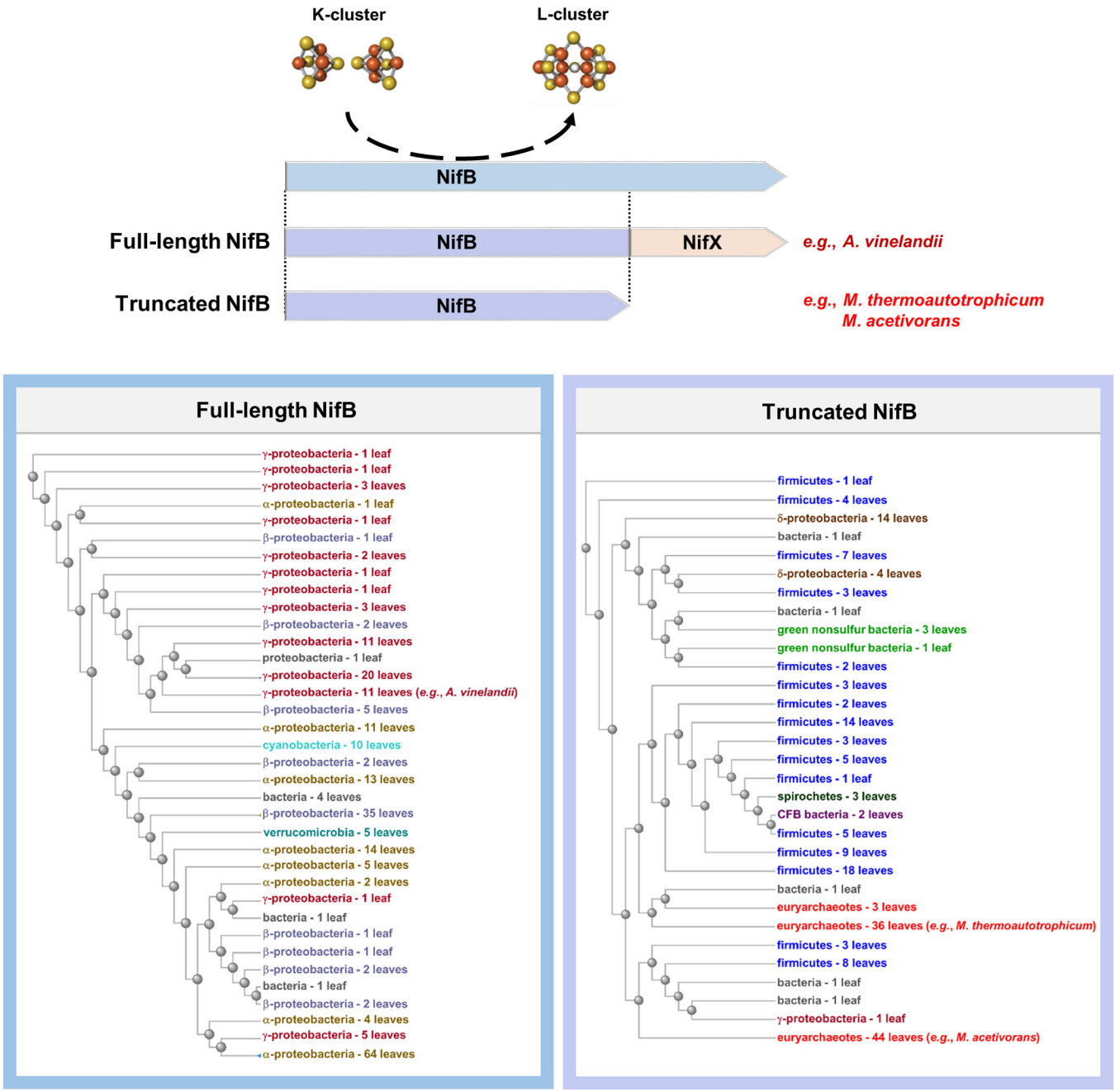

Figure 3. Family of NifB proteins

Phylogenetic analysis divides this protein family into “full-length” (*left*) and “truncated” (*right*) NifB proteins based on the presence or absence of the “NifX domain” toward the C-terminus of the sequence.

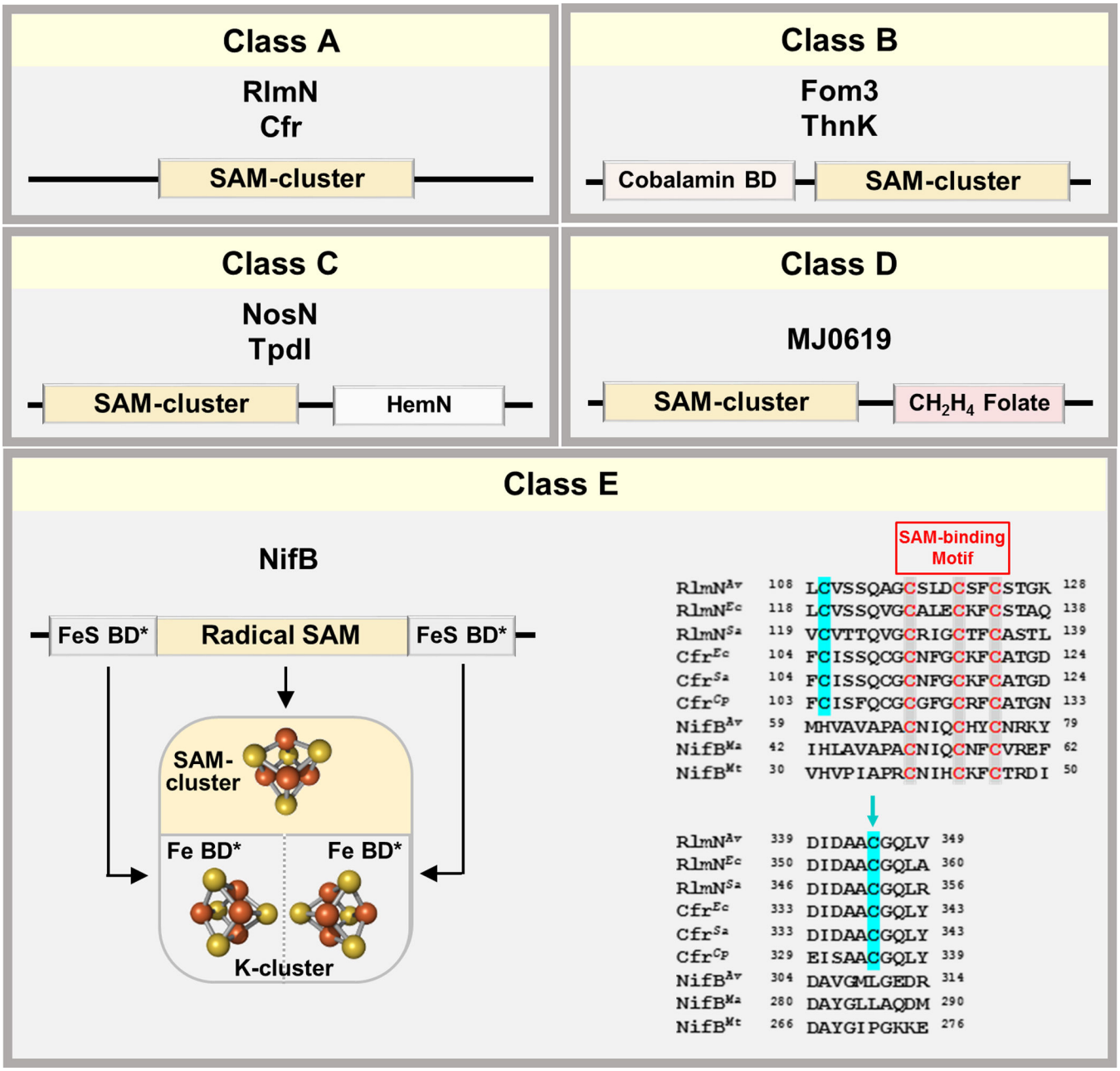

Figure 4. Five classes of radical SAM methyltransferases

(*Upper*) Proteins of classes A–D contain a canonical radical SAM domain and two conserved Cys residues (class A), an N-terminus cobalamin binding domain (class B), a C-terminus HemN domain (class C) and a C-terminus methylenetetrahydrofolate domain (class D), respectively. (*Lower*) Proteins of class E contain a radical SAM domain flanked by conserved Cys and His residues that could potentially serve as FeS cluster-binding domains (*left*). This class of proteins (represented by NifB) do not contain a pair of conserved Cys residues of class A proteins (represented by RlmN and Cfr) based on partial sequence

alignment (*right*). The blue arrow indicates the site of intermediary methylation in Rlm and Cfr (*right*). BD, binding domain.