Divergent Members of the Nitrogenase Superfamily: Tetrapyrrole Biosynthesis and Beyond

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Abstract: The nitrogenase superfamily constitutes a large and diverse ensemble of two-component metalloenzymes. These systems couple the hydrolysis of ATP to the reduction of disparate substrates, from diatomic gases (Mo and alternative nitrogenases) to photosynthetic pigments (protochlorophyllide and chlorophyllide oxidoreductases). Only very recently have the activities of the highly divergent and paraphyletic Group IV nitrogenases begun to be uncovered. This review highlights the first characterized member of this group, which was found to catalyze an unprecedented reaction in the coenzyme F430 biosynthetic pathway, and the catalytic potential of the superfamily that has yet to be explored.

1. Introduction

The nitrogenase complex is the key enzyme system of biological nitrogen fixation, the process by which atmospheric dinitrogen is converted to bioavailable ammonia.[1] The reduction of the triple bond of dinitrogen is an energetically demanding process that is coupled to the hydrolysis of a minimum of 16 equivalents of ATP and requires the concomitant formation of molecular hydrogen.[2,3]

$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (1)$$

Three distinct nitrogenase isozymes have been identified in diazotrophic microorganisms, which differ in their cofactor content and catalytic activity: Mo-nitrogenase, V-nitrogenase, and Fe-only nitrogenase.[4] Mo-nitrogenase is the most widespread and well-characterized of these systems and consists of two metalloenzyme components, the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase).[5,6] The Fe protein is a homodimer consisting of two NifH subunits that each bind an equivalent of ATP and coordinate a single [4Fe-4S] cluster at the dimeric interface.[7] The MoFe protein is an $\alpha_2\beta_2$ heterotetramer comprised of NifD and NifK subunits.[8,9] Each half of the MoFe protein contains two complex metallocofactors, an [8Fe-7S] P-cluster that accepts electrons from the Fe protein and transfers them to a [7Fe-9S-Mo-C-homocitrate] cluster (the FeMo-cofactor), which is the site of N_2 reduction.[10,11]

Dedicated machinery is required for the biosynthesis and insertion of the FeMo-cofactor.[12] Specifically, a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily, NifB, is required for carbide insertion and the formation of the FeMo-cofactor precursor, NifB-co, which is subsequently transferred to a tetrameric scaffold protein, NifEN.[13-15] NifE and NifN are homologous to the NifD and NifK subunits of nitrogenase, respectively, and are thought to have arisen from a paralogous gene duplication and divergence event leading to the efficient assembly of Mo-nitrogenase.[16,17]

The so-called alternative nitrogenases (V-nitrogenase and Fe-only nitrogenase) are comprised of components analogous to the Fe and MoFe proteins.[18] However, the VFe and FeFe proteins, $(VnfDKG)_2$ and $(AnfDKG)_2$, contain an additional γ-subunit and utilize FeV- and FeFe-cofactors in lieu of FeMo-co, respectively.[19-21] Despite their similarities, the catalytic activities of the three nitrogenase isozymes (with either N_2 or alternative substrates such as C_2H_2 or CO_2) vary considerably.[4,22] For example, Mo-nitrogenase is the most efficient at reducing N_2. Conversely, dinitrogen reduction by V-nitrogenase occurs with a greater electron flux towards hydrogen evolution, while the Fe-only nitrogenase was recently shown to have the highest CO_2 reduction efficiency.[23,24] These differences in reactivity highlight the potential catalytic versatility of the nitrogenase superfamily.

Genes encoding proteins with homology to the structural subunits of nitrogenase are widely distributed among bacterial and archaeal phyla, including many species that lack the capacity to fix nitrogen.[25] Blankenship and coworkers examined the phylogenetic history of the nitrogenase superfamily and segregated the homologs into distinct clades (Groups I-V).[26] Group I nitrogenases include the well-characterized Mo-nitrogenases from proteobacteria and cyanobacteria, while Group II includes Mo-nitrogenases from predominantly anaerobic microorganism (such as clostridia, acetogenic bacteria, and methanogens) that are characterized by an ~50 amino acid insertion in the NifD subunit.[27,28] The alternative nitrogenases (V-nitrogenase and Fe-only nitrogenase) belong to Group III. Group IV constitutes a diverse paraphyletic group that includes largely uncharacterized Nif-like proteins (NflH and NflD) from methanogens and certain (typically photosynthetic) nitrogen-fixing bacteria.[29] Group V is comprised of nitrogenase homologs that participate in the biosynthesis of the photosynthetic pigments chlorophyll (dark-operative protochlorophyllide oxidoreductase, DPOR) and bacteriochlorophyll (chlorophyllide *a* oxidoreductase, COR) (Figure 1).[30]

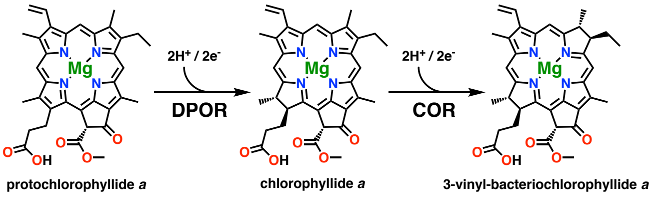

Figure 1. Reactions catalyzed by the Group V nitrogenase homologs DPOR and COR.

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Like nitrogenase, DPOR is a two-component metalloenzyme with a homodimeric Fe protein-like reductase (encoded by the *bchL* gene) and a heterotetrameric catalytic protein (encoded by the *bchNB* genes).[31] Each half of the $(NB)_2$ protein coordinates a [4Fe-4S] cluster with an unusual 3 Cys/1 Asp coordination environment at a position analogous to the P-cluster of nitrogenase.[32] Likewise, the tetrapyrrolic substrate, protochlorophyllide *a*, binds in place of the FeMo-cofactor.[33] DPOR catalyzes the stereospecific 2-electron reduction of the C17=C18 double bond of the D ring of protochlorophyllide *a* (Figure 1).

Similarly, COR catalyzes the stereospecific 2-electron reduction of the C7=C8 double bond of the B ring of chlorophyllide *a* using reductase and catalytic components encoded by the *bchX* and *bchYZ* genes, respectively (Figure 1).[34] The $(X)_2$ and $(YZ)_2$ proteins each coordinate a [4Fe-4S] cluster with 4 Cys ligands and have binding sites for 2 ATP molecules and the chlorophyllide *a* substrate, respectively.[35] Thus, it is clear that members of the nitrogenase superfamily have evolved the capacity to catalyze the ATP-dependent reduction of a wide range of substrates, from small diatomic gases to large organometallic cofactors.

2. Group IV: Coenzyme F430 Biosynthesis

The divergent *nflH* and *nflD* genes are strictly conserved in all methanogens, with the latter a recognized marker of methanogenesis (methanogenesis marker 13). [26,29] Phylogenetic analysis shows that these sequences lie basal in the tree of the nitrogenase superfamily and are positioned between the Nif and Bch clades.[26] The *nflH* and *nflD* genes were shown to be constitutively expressed in the hyperthermophilic non-diazotrophic methanogen *Methanocaldococcus jannaschii* and the encoded proteins were found to associate with one another via a bacterial two-hybrid study and immunoprecipitation.[29] These data suggest that NflH and NflD may play an indispensible role in methanogenesis.

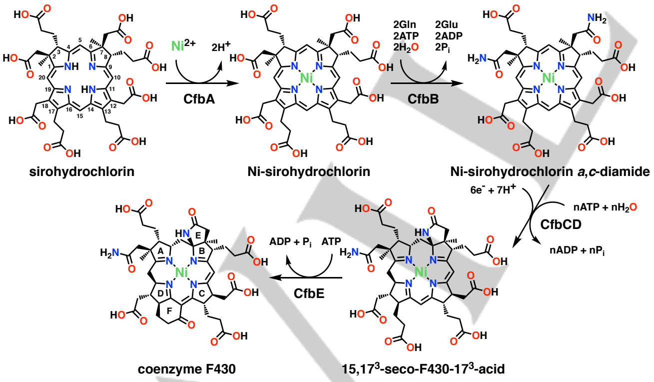

Figure 2. The coenzyme F430 biosynthetic pathway.

Indeed, Zheng et al. demonstrated that NflH and NflD together catalyze a key step in the coenzyme F430 biosynthesis pathway (Figure 2) and redesignated these gene products CfbC and CfbD, respectively.[36] Coenzyme F430 is the unique Ni-containing prosthetic group of methyl-coenzyme M reductase (MCR, the terminal enzyme of methanogenesis) and the most highly reduced tetrapyrrole in Nature.[37,38] CfbCD is responsible for attaining this highly reduced state by catalyzing the ATP-dependent 6-electron reduction of the isobacteriochlorin ring system of the biosynthetic intermediate Ni-sirohydrochlorin *a*,*c*-diamide.[36]

The *cfbCD* genes from *Methanosarcina acetivorans* were heterologously expressed in *Escherichia coli* along with the iron-sulfur cluster (*isc*) biosynthetic genes from *Azotobacter vinelandii*.[36] The CfbCD complex was then purified under strict anaerobic conditions using immobilized metal affinity chromatography (IMAC) via a N-terminal His-tag on the CfbC subunit and assayed using enzymatically synthesized Ni-sirohydrochlorin *a*,*c*-diamide.[36] In the presence of ATP, an ATP regeneration system, and the non-physiological reductant sodium dithionite, CfbCD was found to facilitate the conversion of this substrate to 15,17^3-seco-F430-17^3-acid, a previously identified intermediate in coenzyme F430 biosynthesis.[36,39] The conversion of Ni-sirohydrochlorin *a*,*c*-diamide to 15,17^3-seco-F430-17^3-acid is an unprecedented transformation that involves (in addition to the 6 electron macrocyclic ring reduction) the cyclization of the *c*-acetamide side chain to the γ-lactam E ring and the formation of 7 stereogenic centers (Figure 1).

This result was subsequently confirmed by Moore et. al with individually purified and chemically reconstituted His-tagged CfbC and CfbD from *Methanosarcina barkeri*, using an excess of ATP and Ni-sirohydrochlorin *a*,*c*-diamide biosynthesized in and purified from *E. coli*.[40] Moore et. al also observed that a distinct product with an identical reverse phase high-performance liquid chromatography (HPLC) retention time and observed mass, but with a different UV-visible absorption spectrum, is initially formed, which is slowly converted to 15,17^3-seco-F430-17^3-acid.[40] On this basis, it was proposed that CfbCD only catalyzes the 6-electron reduction of its substrate to form Ni-hexahydrosirohydrochlorin *a*,*c*-diamide (with a double bond at C5=C6 and no E ring), and that the γ-lactamization reaction occurs non-enzymatically.[40] However, the exact structure of the immediate product of CfbCD was not determined. Thus, it is unclear whether an as yet to be identified cyclase is required for the installation of the E ring of coenzyme F430 *in vivo*.

The high-resolution structures of CfbC and CfbD have yet to be determined. Based on the high degree of sequence homology with the Fe protein of nitrogenase, it is expected that CfbC also exists as a homodimer with 2 ATP binding sites and an intersubunit [4Fe-4S] cluster. Consistent with these predictions, iron and sulfide analysis shows the binding of ~2 Fe and S atoms per CfbC monomer, and gel fitration chromatography demonstrated that *apo* CfbC is monomeric, while *holo* CfbC migrates as a dimer.[40] In addition, electron paramagnetic resonance (EPR) spectroscopic analysis of sodium dithionite reduced CfbC is consistent with a $[4Fe\text{-}4S]^{1+}$ cluster whose lineshape is sensitive to the presence/absence of ATP and/or ADP.[40]

In constrast, sequence analysis of CfbD shows that it is a highly divergent member of the nitrogenase superfamily that contains only two conserved Cys residues that occupy positions equivalent to two of the ligands of the P-cluster of nitrogenase. Iron and sulfide analysis, EPR spectroscopy, and gel filtration chromatography also indicate that CfbD contains an intersubunit [4Fe-4S] cluster and can form a dimer in solution.[40] However, unlike every other characterized member of the nitrogenase superfamily, the MoFe protein-like component comprised of CfbD is homomeric rather than heteromeric. Moreover, it is unclear whether the active form of the CfbCD complex contains a $(CfbD)_4$ core and if there are two active sites at the dimeric interfaces of each half (similar to that observed with DPOR) or only a single active site at the center of the tetramer.[33] The latter is a distinct possibility in this case, given the pseudo 4-fold symmetry of the Ni-sirohydrochlorin *a*,*c*-diamide substrate. In view of the lower complexity of its subunit composition and iron sulfur centers, and its basal position within the phylogenic tree of nitrogenase sequences, CfbCD is likely to more closely resemble the primordial 'nitrogenase' present in the last universal common ancestor (LUCA), and suggests that this enzyme may have initially evolved to support methanogenesis (i.e., coenzyme F430 biosynthesis) rather then nitrogen fixation.[17,26]

The activity of nitrogenase is proposed to involve repeated cycles of intercomponent electron transfer, ATP hydrolysis, phosphate release, subunit dissociation, rereduction of the Fe protein, and nucleotide exchange.[41-47] Whether the catalytic cycle of the CfbCD complex follows a similar scheme has yet to be established. The identity of the physiological reductant of CfbCD is also unclear. In the case of nitrogenase, both ferredoxin and flavodoxin have been shown to support N_2 reduction *in vitro* and *in vivo*.[48-50] Currently, no partially reduced intermediates have been detected during the CfbCD-catalyzed reaction, suggesting that the product is not released from the CfbCD complex until it is fully reduced. Thus, it is clear that many mechanistic features of this unique member of the nitrogenase superfamily remain to be elucidated.

3. Divergent Homologs of Unknown Function

The discovery and characterization of divergent homologs of nitrogenase that catalyze the reduction of tetrapyrrolic cofactors highlights the potential unexplored chemical versatility of this important enzyme superfamily. Currently, over 23,600 sequences belonging to the nitrogenase component 1 type oxidoreductase protein family (PF00148) are available in the UniProtKB database. Figure 3 shows a sequence similarity network (SSN) of this protein family. SSNs are multidimensional networks that show pairwise sequence similarity relationships among homologous proteins. In these networks, protein sequences are represented by nodes (circles), which are connected by edges (lines) if their pairwise sequence similiarity exceeds a given threshold. The SSN of the nitrogenase superfamily was generated with the Enzyme Function Initiative Enzyme Similarity Tool (EFI-EST), using a minimum edge alignment score of 35 and filtered to remove partial sequences (less than 250 amino acids) and those resulting from possible gene fusion events (greater than 650 amino acids).[51-54] The chosen edge alignment score threshold is the minimum required to separate the clusters of nodes corresponding to tetrapyrrole biosynthetic enzymes from those of authentic nitrogenases. Given this modest edge alignment score, only the most highly divergent nitrogenase sequences separate into distinct clusters. In particular, the nodes corresponding to CfbD and the subunits of DPOR (BchNB) and COR (BchYZ) all form individual clusters, indicating that their sequences are as different from each other as they are from the subunits of nitrogenase. In contrast, the α subunits from each of the isoforms of nitrogenase (NifD, VnfD, and AnfD) and the NifE subunit of the FeMo-co biosynthesis protein are all found in the same cluster. Likewise, the β subunits (NifK, VnfK, and AnfK) are clustered together with the NifN protein. Thus, not all of the clusters in this SSN are isofunctional. (To completely separate the NifEN sequences from those of nitrogenase requires an edge alignment score of 118, at which point the network separates into well over 100 distinct multi-node clusters.) Regardless, one of the most striking features of this SSN is the large number of additional unique clusters composed entirely of uncharacterized and highly divergent homologs of nitrogenase. Comparing this analysis with the Group designations of Blankenship and coworker, Group I-III nitrogenases form 2 distinct clusters (one that is NifD-like and the other NifK-like), while the Group V nitrogenases form 4 separate clusters (one for each of the structural subunits of DPOR and COR).[26] The greatest diversity thus lies in the Group IV nitrogenases, which contain more than a dozen clusters that are distinct from that corresponding to CfbD. The genes encoding many of these homologs, including those labeled A-A′ through E-E′, are found together in the same gene clusters as *nifDK*-like pairs. Thus, each pair A-A′, B-B′, etc. are predicted to form a heteromeric MoFe-like protein of unknown function.

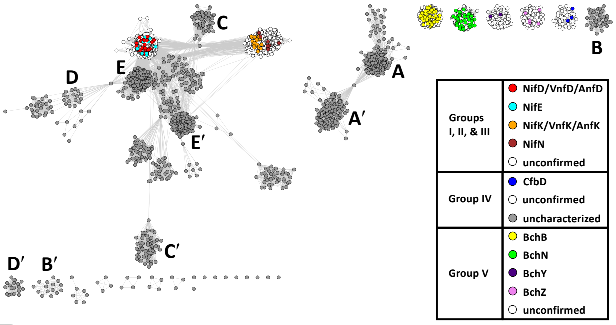

Figure 3. Sequence similarity network (SSN) of the nitrogenase component 1 type oxidoreductase protein family (PF00148). Colored nodes correspond to protein sequences of known function: NifD/VnfD/AnfD (red), NifE (cyan), NifK/VnfK/AnfK (orange), NifN (brown), CfbD (blue), BchB (yellow), BchN (green), BchY (indigo), BchZ (violet). White nodes are comprised of proteins that likely belong to one of these groups, but whose functions have yet to be confirmed. Gray nodes consist of uncharacterized and highly divergent Group IV nitrogenase homologs of unknown function. The clusters of gray nodes labeled A-A′ through E-E′ corresponding to different uncharacterized subgroups discussed in the text. The SSN was generated using an alignment score of 35 and only included protein sequences with lengths between 250 and 650 amino acids.

The clusters of nodes labeled A and A′ are widely distributed in Nature, being found among a large number of archaeal (Crenarchaeota, Euryarchaeota) and bacterial (Actinobacteria, Chlamydiae, Chloroflexi, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes) phyla. Examination of the genomic context of the genes encoding these proteins using the EFI Genome Neighborhood Tool (GNT) shows that they tend to cluster together with homologs of *nifH* and [NiFe] hydrogenase maturation factors (*hypCDEF*).[51-55] This co-occurrence was noted in a previous study by Dixon and coworkers that examined the distribution of nitrogenase-like sequences among microbial genomes and it was suggested that this nitrogenase homolog may be playing a role in the assembly of the active site metallocluster of a hydrogenase.[25] However, given the low sequence similarity to nitrogenase and the lack of conserved residues that coordinate the FeMo-cofactor, another intriguing possibility is that this nitrogenase homolog may bind an alternative hydrogenase-like metallocluster containing diatomic CN ligands and catalyze an unknown ATP-dependent redox reaction.

The clusters of nitrogenase sequences labeled B and B′ are found exclusively in the Elusimicrobia and some Proteobacteria. Genes encoding these homologs are generally clustered together along with a homolog of *nifH*, which is sometimes fused to a *nifD*-like sequence. Interestingly, multiple copies of these genes are frequently present in the same gene cluster, together with several genes encoding members of the radical SAM superfamily.[13] These include homologs of uncharacterized peptide/protein modifying enzymes (His-Xaa-Ser system radical SAM maturases, HxsB and HxsC, and anaerobic sulfatase maturating enzyme, AslB) and a putative radical SAM enzyme containing a N-terminal ferredoxin-like domain and a central carbamoyltransferase-like domain.[56] The functions of these putative enzymes are unknown, but it is again tempting to speculate that they may be involved in the formation of a unique (potentially protein-derived) cofactor within the active site of a B-B′ complex. Alternatively, these genes may be involved in the biosynthesis of a (possibly peptide-based) small molecule that serves as the substrate of this divergent nitrogenase homolog.

Modified peptides (e.g., non-ribosomal peptides (NRPs) and ribosomally synthesized and post translationally modified peptides (RiPPs)) have a wide range of biological functions, including serving as redox active coenzymes, bioactive natural products, and metallophores.[57] Metallophores, such as siderophores (Fe) and chalkophores (Cu), are small molecules secreted by bacteria and archaea that bind trace metals.[58] The metallated metallophores are then taken up through a dedictated import system, which in Gram-negative bacteria typically consists of an outermembrane receptor, a periplasmic binding protein, and an ATP-dependent inner membrane transporter.[59] Once inside the cell, the metal ion is released from the metallophore. This process is often mediated by reduction of the metal center, although in most cases the specific metallophore reductases are unknown.[60] The genes encoding the nitrogenase superfamily members labeled C and C′ are frequently clustered together along with metallophore transporter genes (in addition to homologs of *nifH* and flavodoxin). It is therefore possible that these nitrogenase homologs could bind and catalyze the reduction of such metal-metallophore complexes. The association of nitrogenase homologs with ABC transporter genes was also discussed in the previous study by Dixon and coworkers, where it was noted that these proteins lack FeMo-co ligands and was suggested that they may be playing a role in coupling metal transport to metallocofactor assembly.[25]

The genes encoding the D and D′ nitrogenase homologs, which are found exclusively in methanogenic archaea, also typically cluster with a transporter gene, in this case a homolog of a TrkH-like cation transporter.[61] In addition, the gene cluster usually contains genes encoding a rubredoxin, a flavodiiron protein (FDP), and phosphoribulokinase (PRK). FDPs generally exhibit nitric oxide and/or oxygen reductase activities in a reaction that usually involves the participation of rubredoxin.[62] PRK was recently proposed to participate in a carbon metabolic pathway in methanogens involving ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) termed the reductive hexulose-phosphate (RHP) pathway.[63] What function, if any, this nitrogenase homolog has in the putative RHP pathway is unclear. However, since the FDP may play a role in the response to and detoxification of O_2, one possibility is that the nitrogenase homolog could be involved in the reduction of the oxygenation product of RuBisCO (e.g., the conversion of phosphoglycolate to acetate).

RuBisCO-like proteins have also been implicated in aerobic and anaerobic methionine salvage pathways (MSPs) for 5′-methylthioadenosine (MTA) detoxification and sulfur salvage.[64] Recently, an exclusively anaerobic MSP was identified and partially characterized that couples MTA metabolism to ethylene formation.[65] In this pathway, a class II aldolase converts the common MSP intermediate 5-(methylthio)ribulose-1-phosphate to dihydroxyacetone phosphate and 2-(methylthio)acetaldehyde, the latter of which is sequentially reduced to produce ethylene and (presumably) methanethiol via a 2-(methylthio)ethanol intermediate by an unknown reductase.[65] The nitrogenase sequences designated E and E′ are homologous to the *nifD* and *nifK* genes, respectively, and cluster with sulfur metabolism genes (such as *O*-acetyl homoserine sulfhydrolase and cysteine synthase), as noted previously by Dixon and coworkers.[25] Thus, it is tempting to speculate that these nitrogenase homologs could catalyze the above hypothetical reduction reaction(s) in the anaerobic MSP. However, it was recently demonstrated that *Endomicrobium proavitum*, whose genome only contains a single E-E′ type nitrogenase (along with homologs of *nifH* and *nifB* but no *nifE* or *nifN*), is capable of nitrogen fixation, suggesting that these enzymes bind a FeMo-co-like cofactor and are likely to be competent in N_2 reduction.[66] Nevertheless, an ability to reduce N_2 does preclude that these enzymes are able to reduce other substrates (perhaps preferentially) *in vivo*.

4. Summary and Outlook

The Mo- and alternative nitrogenases have evolved to catalyze an energetically demanding reaction of crucial importance to life on Earth, the ATP-dependent reduction of inert, atmospheric dinitrogen gas to bioavailable ammonia. The discovery and characterization of homologs of nitrogenase catalyzing key steps in the biosynthesis of tetrapyrroles such as chlorophyll and coenzyme F430 have begun to illuminate the functional diversity of this important enzyme superfamily. The rapid increase in genomic and proteomic information has shown that this diversity is likely to be even greater still, with homologs of nitrogenase potentially binding a variety of cofactors/substrates and catalyzing a wide range of unfavorable redox reactions driven by the hydrolysis of ATP. The biological functions of these divergent members of the nitrogenase superfamily are completely unknown, although their genomic contexts suggest some may play roles in metal acquisition, carbon fixation, and sulfur metabolism. Future genetic and enzymological investigations will help elucidate the extent to which Nature has exploited the catalytic potential of these versatile two-component metalloenzyme systems.

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Keywords: coenzyme F430 • nitrogenase • methanogenesis • methyl-coenzyme M reductase • tetrapyrroles

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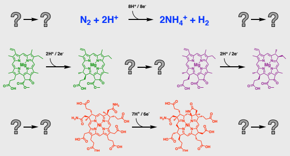
MINIREVIEW



The Group IV nitrogenases constitute the largest and most diverse branch of the nitrogenase superfamily. The first characterized Group IV nitrogenase was shown to catalyze an unprecedented reaction in the coenzyme F430 biosynthetic pathway; however, the activities of the other members of this paraphyletic group are unknown. Bioinformatic analysis suggest that these enzymes may have diverse functions ranging from metal acquisition to carbon fixation and sulfur metabolism.

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