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Iron-Only and Vanadium Nitrogenases: Fail-Safe Enzymes or Something More?

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Abstract

The enzyme molybdenum nitrogenase converts atmospheric nitrogen gas to ammonia and is of critical importance for the cycling of nitrogen in the biosphere and for the sustainability of life. Alternative vanadium and iron-only nitrogenases that are homologous to molybdenum nitrogenases are also found in archaea and bacteria, but they have a different transition metal, either vanadium or iron, at their active sites. So far alternative nitrogenases have only been found in microbes that also have molybdenum nitrogenase. They are less widespread than molybdenum nitrogenase in bacteria and archaea, and they are less efficient. The presumption has been that alternative nitrogenases are fail-safe enzymes that are used in situations where molybdenum is limiting. Recent work indicates that vanadium nitrogenase may play a role in the global biological nitrogen cycle and iron-only nitrogenase may contribute products that shape microbial community interactions in nature.

Contents

INTRODUCTION

The global biological nitrogen cycle is driven by the reduction of atmospheric nitrogen gas (N_2) to ammonia (NH_3) (27, 97), a form of nitrogen that can be used directly by many organisms and is easily converted to other forms of nitrogen such as nitrate (NO_3^-), the preferred nitrogen source for plants. Biological nitrogen fixation is catalyzed by nitrogenase, a complex oxygen-sensitive metalloenzyme (10). The reduction of the triple bond of N_2 is an extremely difficult reaction and is solely a microbial process that is carried out by selected bacteria and archaea. All nitrogen-fixing microbes synthesize a molybdenum (Mo) nitrogenase that has a unique iron (Fe)-Mo cofactor at its active site. Mo nitrogenase is a two-component enzyme consisting of an Fe protein (dinitrogenase reductase, encoded by *nifH*) and a MoFe protein (dinitrogenase, encoded by *nifDK*). The MoFe protein is a heterotetramer consisting of two α subunits and two β subunits (**Figure 1*a***).

The overall reaction catalyzed by Mo nitrogenase is

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i.$$

From this formulation one can see that nitrogen fixation is an ATP-intensive reaction, and it also requires large amounts of protons and reductant in the form of energy-rich electrons. N_2 reduction to NH_3 is difficult to achieve because although it is thermodynamically favorable, there is a large activation energy barrier to catalysis that must be overcome (90) (**Figure 2**). Because of the complexity of the reaction it catalyzes, nitrogenase has a slow turnover rate (104). For these reasons it needs to be synthesized in large amounts, in some cases as much as 10% of total cellular protein (20), to sustain growth under nitrogen-fixing conditions.

In the 1980s it became clear that the nitrogen-fixing bacterium *Azotobacter vinelandii* could express forms of nitrogenase that did not contain Mo. This led to the discovery and initial descriptions of vanadium (V) and Fe-only nitrogenases (4–6, 12, 23, 77, 85). These enzymes comprise VnfHDK and AnfHDK subunits that are homologous to the NifHDK subunits of Mo

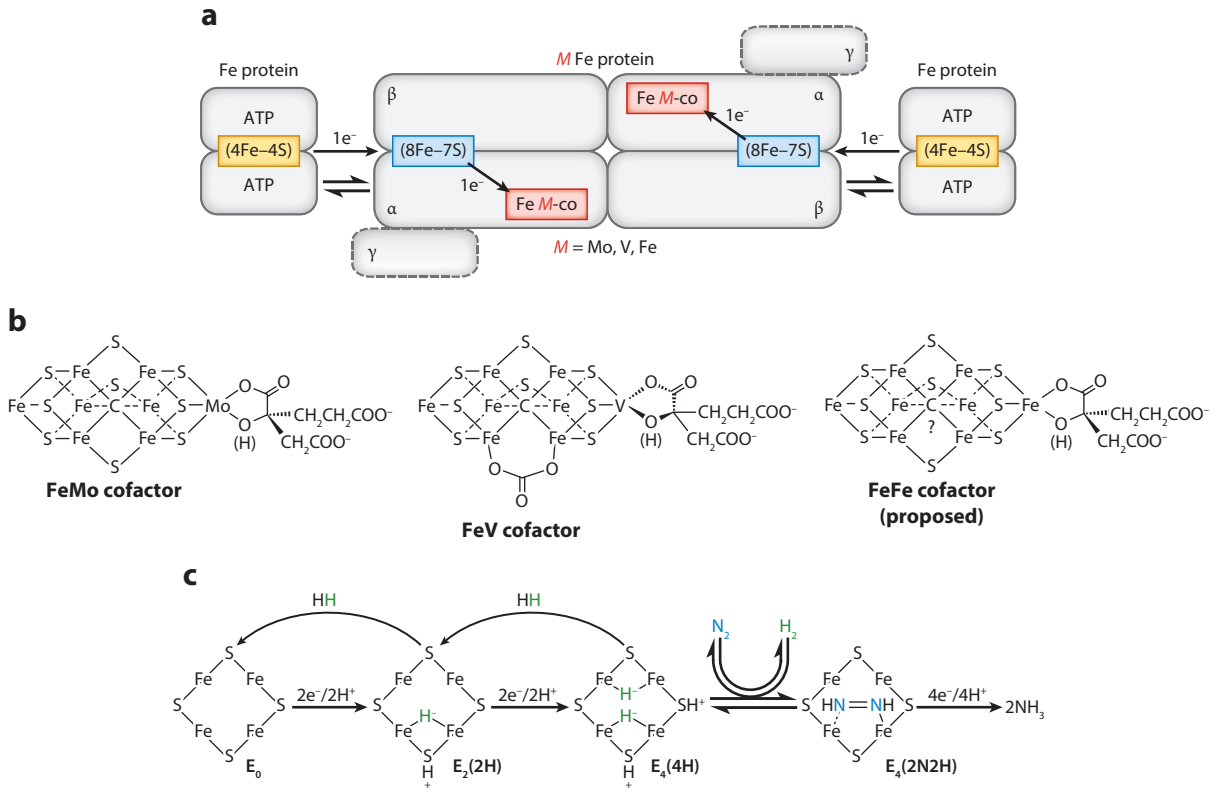

Figure 1

A diagram of the three forms of nitrogenase showing the individual subunits and major route of electron transfer. (*a*) *M* is Mo, V, or Fe. (*b*) The structures for the active site FeMo and FeV cofactors as determined by X-ray crystallography and the predicted structure for the FeFe cofactor are shown. (*c*) The sequence of reactions for hydride reduction at the active site that occurs in all three nitrogenases. Some aspects of the reaction sequence are proposed. Figure adapted from Reference 33.

nitrogenase, but they have either an FeV or an FeFe cofactor at their active sites. The alternative nitrogenases also include VnfG and AnfG subunits as additional components (**Figure 1*a***). So far, alternative nitrogenases have been found only in microbes that also have Mo nitrogenase, most likely because they rely for their cofactor biosynthesis and assembly on Nif proteins that are also necessary for Mo nitrogenase synthesis. V and Fe-only nitrogenases are less efficient and less widespread than Mo nitrogenase (23, 41, 64, 109).

Based on recent studies (32, 33), the reactions catalyzed by the alternative nitrogenases are as follows:

Fe-only nitrogenase,

$$N_2 + 40ATP + 20e^- + 20H^+ \rightarrow 2NH_3 + 7H_2 + 40ADP + 40P_i, \text{and}$$

V nitrogenase,

$$N_2 + 24ATP + 12e^- + 12H^+ \rightarrow 2NH_3 + 3H_2 + 24ADP + 24P_i.$$

All three nitrogenases produce hydrogen gas (H_2) as an obligate aspect of nitrogen fixation. However, the alternative nitrogenases, and in particular Fe-only nitrogenase, produce much more H_2

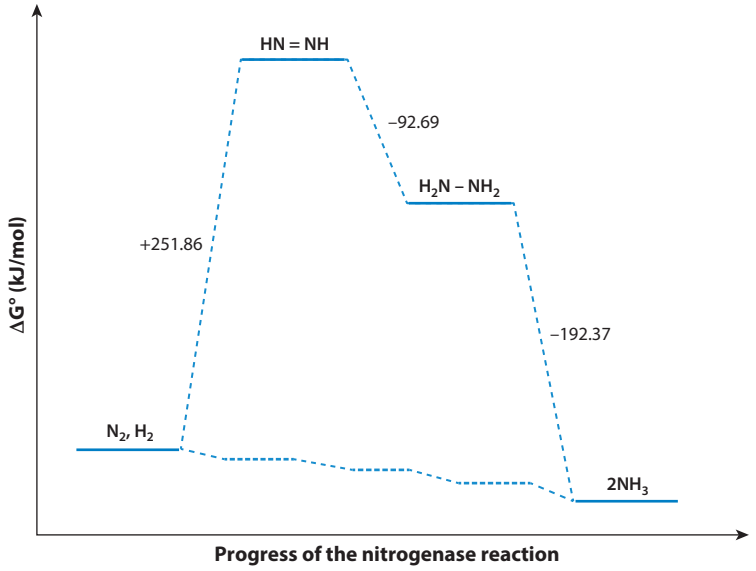

Figure 2

There is a large activation energy barrier to N_2 reduction. Shown is the standard free energy change (ΔG°) between intermediates of the N_2 reduction pathway. Adapted from Reference 90.

per N_2 reduced than does Mo nitrogenase. This has led to the suggestion that nitrogenases might be used to generate H_2 as a biofuel, and there have been a large number of studies directed toward this application (30, 59, 62, 78, 93).

The relative inefficiency of alternative nitrogenases in vitro has led to questions about whether and how much they contribute to global biological nitrogen fixation and whether they may have functions in nature other than nitrogen fixation. Very recent data suggest that V nitrogenase can operate as efficiently as Mo nitrogenase in vivo, at least in some bacteria under some circumstances (53). Nevertheless, the presumption has been that alternative nitrogenases serve a fail-safe role in ensuring that microbes can acquire useable nitrogen when Mo is limiting in the environment, and there is both direct and indirect evidence for this. There are also tantalizing suggestions that alternative nitrogenases could serve other roles in preventing overreduction of bacteria and in carbon conversion. Of the two alternative nitrogenases, the V nitrogenase has been studied much more extensively from a biochemical point of view, and this has provided insights that have illuminated mechanistic features of Mo nitrogenase (96). Fe-only nitrogenase has received much less attention, but recent work has shown that it has unique properties, including the ability to produce significant quantities of methane (CH_4), not shared by the other two nitrogenases (109). Here I discuss what is currently known about Fe-only and V nitrogenase synthesis and their activities against the backdrop of the Mo nitrogenases that operate in the same microbes that encode alternative nitrogenases. The emphasis in this article is on the biological role of nitrogenases and their regulation and activities in vivo.

PHYLOGENETIC DISTRIBUTION AND EVOLUTION

A recent survey of the taxonomic distribution of the three forms of nitrogenase in complete archaeal and bacterial genomes using the *anfD*, *vnfD*, or *nifD* genes as proxies (109) found that among the *Archaea* only hydrogenotrophic methanogens (members of the *Euryarchaeota*) have

nitrogen-fixing representatives. About 18% of euryarchaeotal genomes encode Mo nitrogenase, and 37% of these encode V nitrogenases as well, whereas about 14% encode Fe-only nitrogenase. To date there have been few biochemical or physiological studies of alternative nitrogenases from members of the *Archaea*. Completely sequenced bacterial genomes encompass 34 phyla, with some bacteria in 10 of the phyla encoding *nifD* genes, some in 3 of the phyla encoding *vnfD* genes, and some in 5 of the phyla encoding *anfD* genes. Of 4,342 completely sequenced bacterial genomes, 7.4% encode Mo nitrogenase genes, 10% of these also encode Fe-only nitrogenase, and 4% encode V nitrogenase. The numbers of alternative nitrogenase genes in microbial databases are probably skewed by the relatively large numbers of firmicute and proteobacterial genomes that have been sequenced relative to the rest of the bacteria. Among the firmicutes, *Paenibacillus*, *Clostridia*, and other genera have genes for Fe-only nitrogenase or for both V and Fe-only nitrogenases (61), but members of this phylum are virtually unstudied with respect to the activities and regulation of their alternative nitrogenases. The best-studied alternative nitrogenases are from members of the proteobacteria, especially *A. vinelandii* and several species of purple nonsulfur phototrophs. Besides Mo nitrogenase genes, only V nitrogenase genes have so far been found in *Cyanobacteria*. V nitrogenase genes from the multicellular cyanobacterium *Anabaena variabilis* ATCC 29413 have been studied in some depth (75, 76, 98, 99), and *vnf* genes are also prevalent in species of the closely related genus *Nostoc*, many of which are symbionts of bryophytes including liverworts and hornworts (66).

Some microbes encode all three nitrogenase isozymes. Examples are the hydrogen-utilizing methanogen *Methanosarcina acetivorans* (29), the aerobic gammaproteobacterium *A. vinelandii* (91), and the phototrophic alphaproteobacterium *Rhodopseudomonas palustris* (49). A survey of the genomes of 16 closely related strains of *R. palustris* revealed that different strains have different inventories of alternative nitrogenase genes (74). Whereas all strains encoded Mo nitrogenase and Fe-only nitrogenase, only 13 strains encoded V nitrogenase. In a study of 4 other more distantly related *Rhodopseudomonas* strains, all 4 had Mo nitrogenase genes, and 2 of these also had Fe-only nitrogenase genes (70). This type of uneven distribution of alternative nitrogenase genes in closely related bacteria is consistent with lateral gene transfer events. In fact, there is a recent report of plasmid-borne *vnf* genes in several cyanobacterial symbionts of bryophytes (66).

One group of bacteria in which alternative nitrogenases have so far not been found is root-nodule-forming symbionts of legumes. These include members of the family *Bradyrhizobiaceae* that are symbionts of soybeans and members of the *Rhizobiaceae* that are symbionts of alfalfa, clover, peas, and other leguminous plants (82). It was suggested that these bacteria are unlikely to suffer a Mo shortage because their host plants can acquire and concentrate this trace element from deep soils and even from minerals (73). This would obviate the need for alternative nitrogenases to support plant growth. Another possibility is that the inefficiency of V and Fe-only nitrogenases precludes their use by plant symbionts to support growth of plants.

A number of studies have used the polymerase chain reaction and appropriate primers to identify *nifH* sequences in environmental samples as an indicator of the presence of Mo nitrogenase–containing bacteria (28, 105). However, NifH and VnfH proteins are closely related, with up to 95% amino acid sequence identity, and thus these studies could have unknowingly identified V nitrogenase genes. In recognition of this, a more recent study used *anfD*- and *vnfD*-specific primers to identify alternative nitrogenase genes in sediment and leaf litter from the Florida Everglades and sediments from Sippewissett Marsh (Falmouth, Massachusetts). An analysis of the sequence diversity of *nifD*, *anfD*, and *vnfD* sequences increased the number of known bacterial taxa with alternative nitrogenases by about 20-fold, mostly at the genus and species levels (61).

There have been papers on the evolution of Mo nitrogenase (8, 65, 79), and these analyses have become more refined as more nitrogenase gene sequences have become available and as

tools for inferring protein relationships have improved. In one study that considered the evolution of alternative nitrogenases, Bayesian and maximum likelihood phylogenetic analyses of concatenated NifHDK, VnfHDK, and AnfHDK proteins yielded congruent trees with statistically well-supported lineages for each of the three nitrogenase isozymes (7). There were two distinct Mo nitrogenase sublineages. One comprises proteins solely from hydrogenotrophic methanogenic archaea, and the second is a mix of Mo nitrogenases from both bacteria and archaea. Nestled between the two Nif sublineages is a monophyletic lineage of V nitrogenases, with Fe-only nitrogenases as an offshoot. This analysis supports the ideas that V nitrogenase evolved from Mo nitrogenase, possibly following a gene duplication event, and that Fe-only nitrogenase may have evolved from V nitrogenase (7).

BIOCHEMICAL ASPECTS

Based on protein sequence comparisons, it is clear that all three nitrogenase isozymes have the same basic architecture, with the addition of AnfG and VnfG subunits for the alternative nitrogenases (**Figure 1*a***). The functions of the AnfG and VnfG subunits are unknown, but they are essential for activity. NifH/VnfH/AnfH proteins (dinitrogenase reductases) are about 63 kDa, and NifDK/VnfDK/AnfDK proteins (dinitrogenase) are about 230 kDa. The Mo dinitrogenase reductase is a homodimer that transfers one electron at a time to the MoFe dinitrogenase in a reaction that is coupled to the hydrolysis of two ATPs. Electrons are delivered one at a time from a 4Fe–4S cluster in NifH to an 8Fe–7S metallocluster called the P cluster, in the dinitrogenase reductase (NifDK). Electrons are transferred from the P cluster to the FeMo cofactor that is the site of substrate reduction (38, 39, 89). In the last 38 years, multiple crystal structures of Mo nitrogenase have been obtained that have informed about the compositions and coordination of metal clusters and about features of the FeMo cofactor that are important for catalysis (81). A first structure of V nitrogenase was reported in 2017 (95), and this and other studies showed that the basic design and composition of the FeV cofactor are similar, though not identical, to those of the $MoFe_7S_9C$-homocitrate cofactor (**Figure 1*b***). Amino acid residues that are known to coordinate the 4Fe–4S cluster in the NifH protein and also the P [8Fe–7S] cluster and the FeMo cofactor in the NifDK subunits are conserved in the corresponding VnfHDK subunits. The structure of the FeFe cofactor embedded in AnfD has not been determined, but spectroscopic studies suggest that it is homologous in structure to the FeMo cofactor (48). The FeMo cofactor is synthesized with an FeFe form as a precursor, and then there is a step when a Mo atom replaces one of the Fe atoms. It has been suggested that the FeFe precursor is used as the FeFe cofactor in Fe-only nitrogenase (25).

By contrast with studies of Mo nitrogenases and V nitrogenases, work on Fe-only nitrogenase has lagged behind, and it is only recently that a basic characterization of the mechanism of N_2 reduction by this enzyme has been published (33). This study showed, importantly, that Fe-only nitrogenase purified from *A. vinelandii* does not contain even traces of Mo or V, as some had theorized in the past. It also showed through spectroscopic studies with deuterium (D) that Fe-only nitrogenase makes HD when turned over under N_2 and D_2, and thus it has the same reductive elimination mechanism that operates in Mo nitrogenase. In Mo nitrogenase, four electrons and four protons accumulate on the FeMo cofactor to create a state that contains two Fe-H-Fe-binding hydrides. The two hydrides combine to produce H_2 in a reductive elimination step that is coupled to N_2 binding and reduction (**Figure 1*c***). Although the reductive elimination mechanism nicely explains why one H_2 is produced for each N_2 reduced by Mo nitrogenase, Fe-only nitrogenase makes much more H_2 than Mo nitrogenase, on the order of 6–7 H_2 produced per N_2 molecule reduced. This likely reflects the finding that the K_m for N_2 is fivefold higher for the

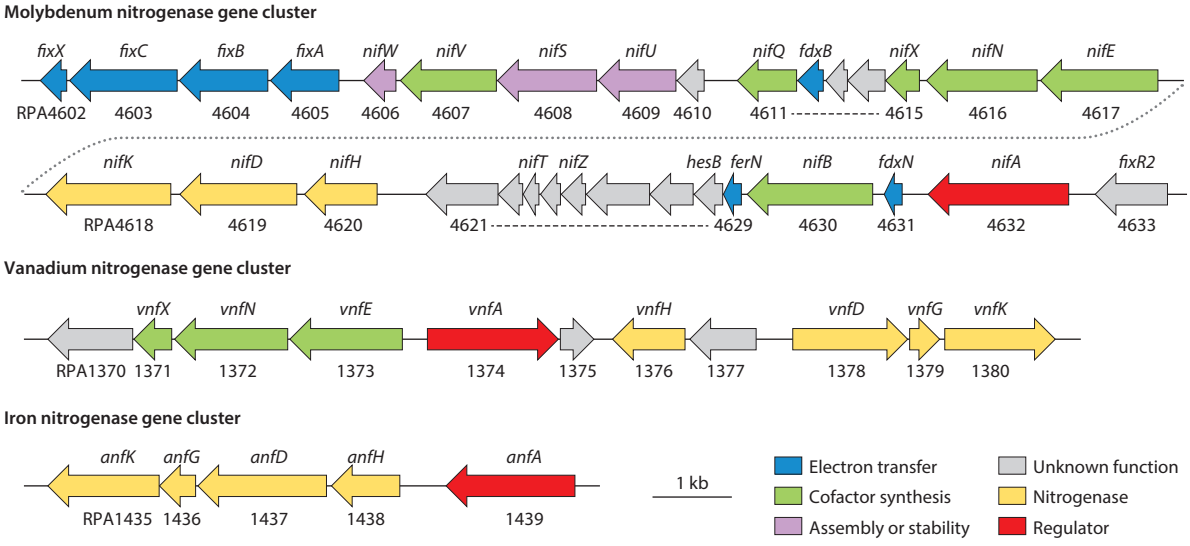

Figure 3

The organizations of *nif*, *vnf*, and *anf* genes encoding the three forms of nitrogenase in *Rhodopseudomonas palustris*. Gene functions were assigned from Rubio & Ludden (87). Adapted from Reference 71.

Fe-only nitrogenase than that for Mo nitrogenase, so that N_2 at one atmosphere of pressure is insufficient to outcompete the hydride protonation that is always catalyzed by nitrogenase in the absence of N_2 to produce H_2. A similar line of evidence explains why V nitrogenase makes less H_2 than Fe-only nitrogenase but more than the Mo enzyme (32).

SYNTHESIS AND ASSEMBLY OF ACTIVE V AND Fe-ONLY NITROGENASES

The minimum dedicated gene set for Mo nitrogenase, including its structural genes, genes for FeMo cofactor synthesis, and genes for enzyme assembly, is considered to be *nifH*, *nifD*, *nifK*, *nifB*, *nifE*, and *nifN* (9, 13, 22, 84) (**Figure 3**). In addition, *nifU* and *nifS* gene products are important for supplying Fe-S clusters, and a *nifV* gene is often present in some microbes and encodes a protein that catalyzes the synthesis of homocitrate, a core component of the FeMo cofactor. However, homocitrate can also derive from general metabolism depending on the species. *nifB* encodes a radical *S*-adenosyl methionine–dependent enzyme that plays a central role in assembling the 8Fe core of FeMo and FeV cofactors (14, 42). NifEN and VnfEN, which are homologous to NifDK and VnfDK, provide scaffolds for the final maturation of the cofactors (9, 94). There are also V and Fe-only nitrogenase–specific regulatory genes. Finally, nitrogenase needs to be provided a source of low-potential electrons in the form of reduced ferredoxin or flavodoxin for activity, and there are several types of enzymes, some of which may be coordinately regulated with *nif* genes, that can accomplish this. These include pyruvate-flavodoxin oxidoreductase (92), an electron-bifurcating FixABCX system (24, 26, 50), and a membrane-bound Rnf system (3).

Generally speaking, *vnfHDGK* structural genes and *vnfEN* assembly genes are found coregulated in a given microbe, and the coregulated genes often include genes for a V transport system as well (71, 76). Structural genes for Fe nitrogenase are clustered together in diverse genomes as *anfHDGK*, but there are no reported instances of *anfEN* assembly genes (**Figure 3**). From this it

has been concluded that some *nif* genes are involved in VFe and FeFe cofactor synthesis and *nif* genes for enzyme assembly are also sometimes needed to produce functional alternative nitrogenases. An elegant demonstration of a minimum set of *nif* genes required for synthesis of Fe-only nitrogenase was obtained by Yang et al. (103), who engineered *Escherichia coli* to synthesize active Fe-only nitrogenase using *anf* genes from *A. vinelandii* and *nif* genes from *Klebsiella oxytoca*. This group found that in addition to *anfHDGK*, *nifUSVB* genes were required for synthesis of functional Fe-only nitrogenase. Interestingly, provision of the scaffold genes, *nifEN* or *vnfEN*, to the reconstituted *E. coli* system did not enhance Fe-only nitrogenase activity, suggesting that they are not required for FeFe cofactor synthesis. It is possible that *anfDK* served this role. These experiments show clearly that genes that are required for synthesis of Mo nitrogenases and that are under the control of Mo nitrogenase regulation are also required for Fe nitrogenase synthesis, thus explaining why Fe-only nitrogenase is only found in microbes that also synthesize Mo nitrogenase. And the same is true to a lesser degree for V nitrogenase, which requires, at the minimum, NifB, as far as is known to date (46, 54).

SUBSTRATE RANGE

An important feature of Mo nitrogenase is that it reduces a broad range of substrates in addition to N_2 and protons. Early work with purified Mo nitrogenase revealed that it reduces the gas acetylene ($HC{\equiv}CH$) to ethylene ($H_2C{=}CH_2$) in a two-electron/two-proton reaction (18). Because of technical difficulties with measuring reduction of N_2 to NH_3, especially in vivo, the acetylene reduction assay has become the standard assay used to measure nitrogen fixation. Beyond acetylene there is a long list of nitrogen-, sulfur-, and carbon-containing small molecules that can be reduced by Mo nitrogenase, many of which are nonphysiological (67, 90). Many of these types of studies were carried out in vitro with purified nitrogenases and with other metal catalysts sometimes present. The substrate profiles of alternative nitrogenases have not been investigated nearly as thoroughly. V nitrogenase provided with carbon monoxide (CO) in vitro produced C_2 and C_3 hydrocarbons (40). V nitrogenase expressed in *A. vinelandii* also reduced and concatenated CO to hydrocarbons (80). Mo nitrogenase, and probably the alternative nitrogenases as well, reduces carbon dioxide (CO_2) to formic acid (47). V and Fe-only nitrogenases reduce CO_2 to CH_4, with Fe-only nitrogenase producing substantially more CH_4 (109).

REGULATION OF ALTERNATIVE NITROGENASE SYNTHESIS AND ACTIVITY

Since nitrogenases require large amounts of ATP and high-energy electrons for catalysis, cells synthesize these enzymes only when absolutely needed to supply usable nitrogen. Accordingly, there is overarching repression of nitrogenase synthesis and activity at transcriptional and sometimes posttranslational levels by fixed nitrogen sources, especially NH_3 (21, 43). Nitrogenase is also extremely sensitive to oxygen, and this may explain why many nitrogen-fixing microbes are obligate anaerobes. However, this is not the case for all nitrogen fixers, and a number of bacteria, depending on the species, control nitrogenase synthesis in response to oxygen (21). Alternative V and Fe-only nitrogenases are often controlled by Mo availability as well (16, 56).

Protection from Oxygen

The number of species for which V or Fe-only nitrogenase regulation has been studied is very small; these include *A. vinelandii* and several species of purple nonsulfur bacteria such as

Rhodobacter capsulatus, *Rhodospirillum rubrum*, and *R. palustris*, all alphaproteobacteria. *A. vinelandii* is fascinating because it is an obligately aerobic bacterium, and growth in oxygen would seem antithetical to the expression of active nitrogenase. It turns out that *A. vinelandii* has several mechanisms to protect nitrogenase from inactivation by oxygen. One is respiratory protection in which high respiratory activity by multiple terminal oxidases has been proposed to keep the cytoplasm, where nitrogenase is located, in an anoxic state. There is some controversy over just how important this mechanism is, but it likely does play some role (72). A second mechanism is one of conformational switch off where a small [2Fe:2S] ferredoxin also termed FeSII or Shethna protein binds to the NifHDK nitrogenase complex to force it into an inactive but oxygen-tolerant state to survive periods of high oxygen (88). Whether this protein can also inactivate the alternative V and Fe-only nitrogenases in response to oxygen stress does not appear to have been tested. The *A. vinelandii anf* gene cluster includes a gene (*avin_49040*, also called *anf3*) that encodes a flavocytochrome that can function as an oxygen-consuming oxidase, suggesting that it may have a specific role in protecting Fe-only nitrogenase from oxygen (101).

Purple nonsulfur proteobacteria are facultative anaerobes that grow aerobically as heterotrophs but anaerobically as phototrophs, in which situation they generate ATP from light energy and grow well under nitrogen-fixing conditions. This suggests that in theory, this group of bacteria should not have a need to protect its nitrogenases from oxygen. However, there is a report of nitrogen fixation by purple nonsulfur bacteria during microaerobic growth (55). Consistent with this, both *R. capsulatus* and *R. palustris* have homologs to Avin_49040, the *A. vinelandii* flavocytochrome encoded by *anf3* that likely functions in oxygen consumption. In *R. palustris* this gene (*rpa1432*) is highly expressed in cells expressing Fe-only nitrogenase but barely detectable in Mo nitrogenase–expressing cells (71). In *R. capsulatus*, *anf3* (*Rcasp_rcc012094*) is required for Fe-only nitrogenase–dependent growth. Both *R. palustris* and *R. capsulatus* also encode homologs of the Shethna protein II (FeSII). This protein (RPA1928) is expressed at high levels in *R. palustris* strains growing using any one of the three nitrogenases (71).

Regulation by Nitrogen Availability

In free-living proteobacteria, nitrogenase gene expression is controlled by a hierarchy of regulatory proteins that are involved in nitrogen metabolism in general. Hence seminal work on regulation of nitrogen metabolism by non-nitrogen-fixing bacteria like *E. coli* is foundational to understanding how nitrogenase is regulated (100). We know from studies with *E. coli* that free-living proteobacteria sense their intracellular nitrogen status as the ratio between 2-oxogluterate (2-OG) and glutamate, and this is often reflected by the uridylylation state of small trimeric signaling proteins called P_{II} proteins that bind to other proteins to regulate their activities (63). 2-OG can also bind directly to proteins to regulate their activities (44). Many nitrogen-fixing proteobacteria have two or three P_{II} proteins, usually called GlnB and GlnK or GlnB, GlnK1, and GlnK2 (1). A bifunctional uridylyltransferase/uridylyl-removing enzyme called GlnD adds uridylyl (UMP) groups to P_{II} protein when 2-OG levels are high (a sign of nitrogen insufficiency), and it removes uridylyl groups from P_{II}-UMP when 2-OG levels are low and cells have sufficient fixed nitrogen. The activity of the NtrBC two-component regulatory system, consisting of a histidine kinase and response regulator, is controlled by a P_{II} protein, usually GlnB, such that when cells are grown with NH_3 as a sole nitrogen source, P_{II} is not uridylylated, and as such it binds to NtrB to inhibit its autophosphorylation and activate its phosphatase activity. However, under nitrogen-fixing conditions when cells are starved for fixed nitrogen, P_{II}–UMP is formed, and in this form P_{II} does not bind to NtrB, thus allowing it to phosphorylate NtrC. NtrC–P then activates the expression of a variety of genes involved in nitrogen metabolism, including *glnK1* and *glnK2*, NH_3

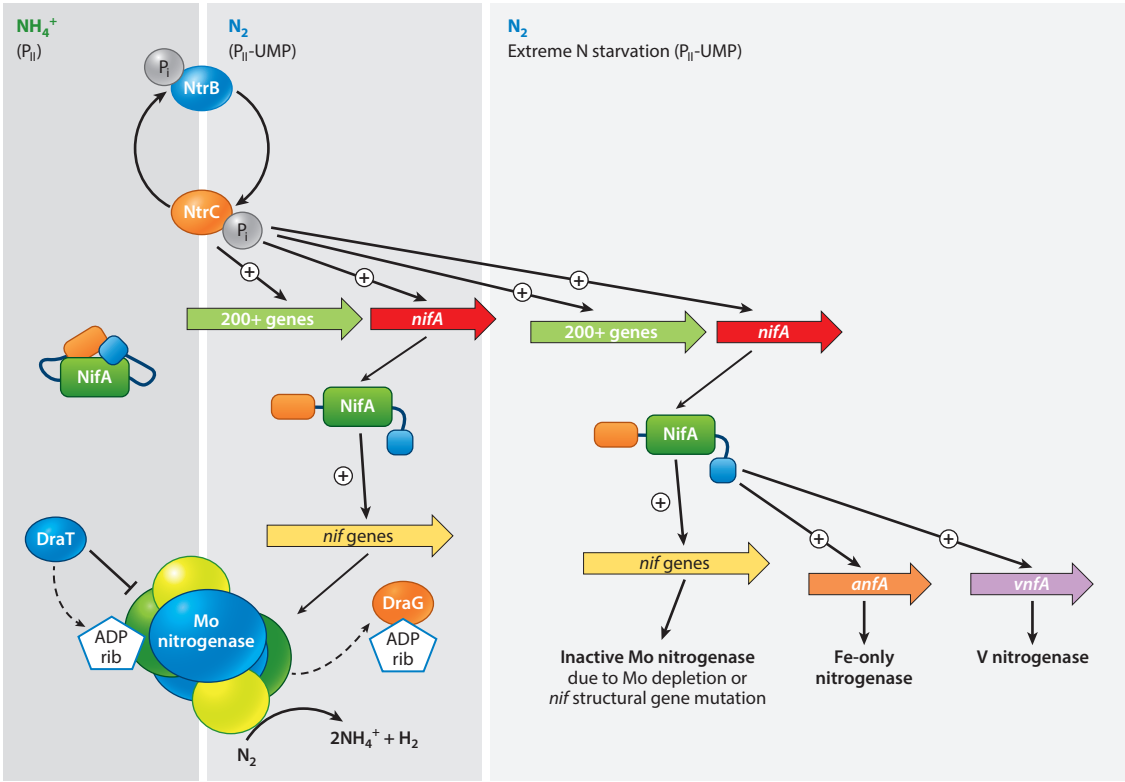

Figure 4

A model for the regulation of synthesis and activities of the three forms of nitrogenase in *Rhodopseudomonas palustris* in response to fixed nitrogen starvation. The predicted activities of regulatory proteins NtrBC, NifA, and DraT in ammonium-grown cells are shown on the left side, whereas the predicted activities of NtrBC, NifA, and DraG in cells that are starved for fixed nitrogen (nitrogen-fixing cells) and expressing Mo nitrogenase are shown in the middle panel. Not shown is how the activities of these proteins are influenced by interactions with P_{II} proteins, which are either unuridylylated or uridylylated, depending on cellular nitrogen status. As shown in the right-hand panel, cells subjected to extreme nitrogen starvation because of an inability to express functional Mo nitrogenase are predicted to express active V nitrogenase (when V is present) as well as Fe-only nitrogenase. Fe-only nitrogenase and V nitrogenase are posttranslationally modified by ADP-ribosylation and inactivated when cells are exposed to ammonium. This reversible reaction occurs as is shown for Mo nitrogenase in the left and middle panels.

transporter genes; the glutamine synthetase gene; and in some cases, *nifA* (21) (**Figure 4**). NifA is an enhancer binding protein that is a master regulator of bacterial nitrogenase gene expression. It has an N-terminal GAF domain, a central AAA+ domain, and a C-terminal helix-turn-helix binding domain (83) (**Figure 5**). In bacteria studied, NifA expression is only slightly higher, threefold or so, in cells growing under nitrogen-fixing conditions (71). In *A. vinelandii*, *nifA* is not controlled by NtrBC but is expressed constitutively (31), and a second protein that is also constitutively expressed, called NifL, posttranslationally regulates the NifA protein by binding to it. In conditions of nitrogen excess, GlnK binds to the NifLK complex to inactivate it (52). Under conditions of high oxygen NifL, which has a PAS domain, also inactivates NifA (36). Thus, when conditions of nitrogen limitation and low oxygen have been met, NifA is active. In other bacteria such as the phototroph *R. rubrum* and probably *R. palustris*, NifA is activated when P_{II}-UMP (GlnB) binds to its N-terminal GAF domain (108). In fact, mutations or a small deletion in the Q-linker region of *R. palustris nifA*, called *nifA** mutations, render NifA constitutively active so that cells express

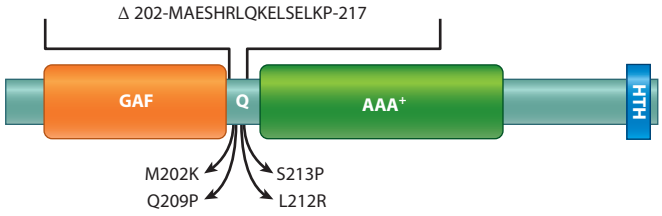

Figure 5

Diagram of the domain structure of the NifA protein. The individual single amino acid changes or the deletion shown, all in the Q-linker region of the protein, renders it active in *Rhodopseudomonas palustris* cells grown with NH_3. Adapted from Reference 58.

nitrogenase and produce H_2 when growing with NH_3 as a sole nitrogen source in the absence of nitrogen gas (58, 83). These Q-linker mutations probably allow NifA to unfold such that it is active at all times (**Figure 5**).

The Role of Mo or Extreme Nitrogen Starvation in Alternative Nitrogenase Expression and Activity

Proteins homologous to NifA, called VnfA and AnfA, control expression of *vnfHDGK* and *anfHDGK* genes in bacteria that have been studied, all of which are proteobacteria. A condition of fixed nitrogen starvation must be met for *vnfA* and *anfA* to be expressed. But in addition, there are regulatory controls exerted by Mo, V, and extreme nitrogen starvation.

An important point is that while it is convenient to discuss the expression of the three nitrogenase isozymes as being mutually exclusive, this is not necessarily the case. In *R. capsulatus*, Mo and Fe nitrogenases are coexpressed under conditions of low Mo, and in *R. palustris* Fe and V nitrogenases are coexpressed when Mo nitrogenase is inactive and V is present (17, 71).

Regulation of nitrogenase synthesis in response to molybdate ion (MoO_4^{2-}) has been extensively studied in *R. capsulatus* (16). Two transcription factors called MopA and MopB are encoded in its nitrogen fixation gene cluster. MopA and MopB are transcriptional repressors similar to ModE from *E. coli*, which has a molybdate-binding domain in its C terminus and a helix-turn-helix DNA-binding domain in its N terminus. Binding of Mo enhances the affinity of MopA and MopB proteins for target promoters that include a DNA consensus sequence Mo-box. One of the genes that is transcriptionally repressed in this fashion is *anfA*. Various other nitrogen-fixing microbes that encode alternative nitrogenases, including *A. vinelandii*; *M. acetivorans*; and the purple nonsulfur bacteria *Phaeospirillum fulvum*, *Rhodobacter maris*, and *Rhodomicrobium vannielii*, all have conserved Mo-boxes in the promoter regions of their *vnfA* (where present) and *anfA* genes or operons (16). This suggests that alternative nitrogenase expression is repressed by Mo availability in these species in a manner similar to that seen in *R. capsulatus*. In the case of *A. vinelandii*, repression of *anfA* and *vnfA* transcription by Mo has been shown directly (77).

The purple nonsulfur bacteria *R. palustris* and *R. rubrum* do not regulate *anfA* transcription in response to Mo but instead synthesize active Fe-only nitrogenase when Mo nitrogenase is not active, due to mutation of its structural genes for example, regardless of the level of Mo in growth medium (51, 71). The same is true of V nitrogenase expression in *R. palustris*. Moreover, the *anfA* and *vnfA* genes are not preceded by a Mo-box, even though a Mo transporter operon is preceded by a Mo-box in *R. palustris* (16). In *R. palustris* VnfA is 70% identical to NifA but AnfA is just 36% identical in amino acid sequence to NifA. When N_2 is supplied as the nitrogen source for growth, *anfA* and *vnfA* genes are upregulated about 70- and 30-fold, respectively, in situations where Mo

nitrogenase is not expressed, and the *anfHDGK* and *vnfHDGK* structural genes are upregulated as well (71). This suggests that AnfA and VnfA proteins are active in transcribing their target genes when they are expressed. Unlike the case of NifA, there is no further need for them to assume an active conformation to become proficient in transcription (**Figure 4**). Available evidence suggests *anfA* and v*nfA* expression are activated under conditions of nitrogen deprivation that are more extreme than those required to activate *nifA* transcription (71). The mechanism for this has yet to be determined, but based on what is known about nitrogen metabolism in general, it could be that elevated 2-OG levels or superuridylylated P_{II} proteins are involved. Transcriptome experiments indicate that NifA activates *anfA* and *vnfA* expression in *R. palustris* (35), but there must be other requirements as well because strains expressing active NifA* protein do not express alternative nitrogenases (83).

Posttranslational Regulation

Upon addition of NH_3 to growth medium or in response to a sudden decrease in energy, some members of the *Alphaproteobacteria* immediately switch off Mo nitrogenase activity. The mechanism for this is a posttranslational ADP-ribosylation of a conserved arginine on NifH (dinitrogenase reductase) (45, 69). This prevents NifH from interacting with NifDK, thereby inhibiting electron transfer and nitrogenase activity. This modification is reversible and catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase–activating glycohydrolase (DraG) enzymes. ADP-ribosylation of AnfH has been reported in the purple nonsulfur bacteria, including *R. capsulatus* and *R. palustris*, and ADP-ribosylation of VnfH also occurs in *R. palustris* (34, 57).

AnfH is much more divergent from NifH than VnfH, with about 45% amino acid identity. But NifH, VnfH, and AnfH in *R. palustris*, at least, each have the conserved arginine that is the site of ADP-ribosylation at the same relative position (34). The *R. palustris anf* gene cluster includes a *draT1* gene (*rpa1431*) that is coordinately regulated with *anfHDGK* genes (71). There is also a *draT2* gene that is expressed constitutively and that mediates switch off Mo nitrogenase activity, and this is paired with a *draG2* gene on the chromosome (49, 71). The DRAG-DRAT system of nitrogenase posttranslational modification is controlled by P_{II} proteins in all cases studied (**Figure 4**).

ACTIVITIES OF V- AND Fe-ONLY NITROGENASES IN NATURE

Lack of information about the contribution of alternative nitrogenases to nitrogen fixation in nature impedes a complete understanding of the biological nitrogen cycle. There is plenty of indirect evidence that V and Fe-only nitrogenases are probably active. Mo is the scarcest micronutrient in the Earth's crust (102), and bacteria that encode alternative nitrogenases always seem to be able to express functional enzyme when grown in the laboratory under conditions of Mo limitation. In addition, alternative nitrogenase genes have been reported to be expressed in cyanobacterial symbionts of lichens (37), in termite hindguts (68), and in soil microcosms amended with V (2).

A problem with assessing alternative nitrogenase activity directly has been that until recently, there was no easy approach to measure and distinguish the activities of the Mo and alternative nitrogenases in environmental samples. A valuable new method is the isotope acetylene reduction assay (ISARA), which derives from the observation that the three nitrogenases fractionate the natural abundance of $^{15}N/^{14}N$ to different degrees, with alternative nitrogenases significantly decreasing the $^{15}N/^{14}N$ ratio compared to Mo nitrogenase (107). It turns out that Mo and alternative

nitrogenases also differ in fractionating the abundance of $^{13}C/^{12}C$ of acetylene in the acetylene reduction assay (106). This can be measured by examining the isotopic abundance of ^{13}C ethylene, the product of acetylene reduction by nitrogenase, by gas-chromatography-combustion-isotope ratio mass spectrometry. Another diagnostic feature of alternative nitrogenases is that they catalyze the production of ethane along with ethylene as a product of acetylene reduction, with Fe-only nitrogenase producing more ethane than V nitrogenase (19, 71). Mo nitrogenase, on the other hand, reduces acetylene exclusively to ethylene.

In practice it is necessary to have fairly high nitrogenase activity to be able to detect ethane or to make use of ISARA, and this can be a limitation for broad environmental surveys. However, there are environments where these assays have provided evidence for alternative nitrogenase activities. In one study ISARA measurements of leaf litter samples from Everglades National Park and sediment samples from Great Sippewissett Marsh suggested the presence of active alternative nitrogenases, and these samples also contained *anfD* and *vnfD* genes (61). These measurements likely included contributions from the activities of all three nitrogenases in the sediment samples, and thus it was only possible to conclude that Mo nitrogenase alone could not account for the acetylene carbon isotope fractionation values obtained, and that some portion would have to have been contributed by Fe-only and V nitrogenases. The authors estimated that 24% and 18% of the acetylene reduction measured in leaf litter samples and marsh sediments, respectively, was due to alternative nitrogenase activities.

A second, more extensive study examined the contribution of V nitrogenase to biological nitrogen fixation in cyanolichens in boreal forests (15). Boreal forests occupy the subarctic and are dominated by plants such as birch, poplar, and conifers that are not known to host nitrogen-fixing symbionts. These forests and their soils store a substantial amount of carbon, and the availability of fixed nitrogen has been proposed to be a factor that could constrain their ability to absorb future human-made increases in atmospheric CO_2. Moreover, cyanolichens have been reported to contribute over 85% of fixed nitrogen to a subarctic birch forest (86). Here cyanolichens are a symbiotic association between a fungus and a cyanobacterium, commonly *Nostoc*, a genus known to express active Mo and V nitrogenases. In a sampling of boreal cyanolichens across a longitudinal transect in eastern Canada, half of the samples had alternative nitrogenase activity. Since cyanobacteria are known to encode V but not Fe-only nitrogenase, the authors concluded that all the activity measured was due to the V alternative nitrogenase. The Mo content of the vegetative part of the lichen (the thallus) was measured and found to be inversely related to the average V nitrogenase activities. These results suggest that as much as 50% of total cyanolichen nitrogen fixation could be due to the activity of V rather than Mo nitrogenases. One anticipates that other studies will be carried out to evaluate the contribution of alternative nitrogenases to global nitrogen fixation.

POSSIBLE ROLES FOR NITROGENASES IN ADDITION TO NITROGEN FIXATION

The extreme energetic requirements for nitrogen fixation are superficially reflected in the requirements for the Haber-Bosch industrial process for converting N_2 to NH_3 for fertilizer (11). This process requires 200 atmospheres of pressure and 400°C to convert $N_2 + H_2$ to NH_3 using an Fe catalyst. That this reaction is accomplished by microbes living at 1 atmosphere of pressure and at 30°C is remarkable. At the same time and as a consequence of their complexity, nitrogenases catalyze reactions in addition to nitrogen fixation. The most predominant one is H_2 production. There is an important role for H_2 production in oxidizing electron carriers such as NAD or

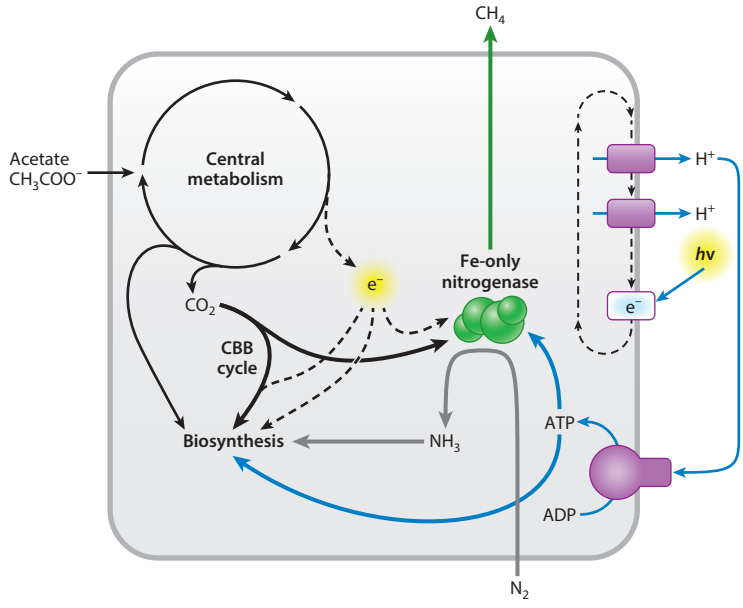

Figure 6

A depiction of a *Rhodopseudomonas palustris* cell expressing active Fe-only nitrogenase that is producing NH_3, CH_4, and H_2 (not shown) as products. Cells are growing under anaerobic phototrophic conditions, such that they generate ATP from light by cyclic photophosphorylation. Acetate, provided as the carbon source, is used for biosynthesis and as an electron donor to nitrogenase. Excess electrons can be combined with CO_2 and converted to cell biomass by the Calvin-Benson-Bassham (CBB) cycle for CO_2 fixation. The symbol *hν* is used to depict energy input from light. The differing arrow colors refer to the different inputs and outputs from Fe-only nitrogenase as shown in the figure. Adapted from Reference 109.

ferredoxin that become reduced during metabolism, especially during anaerobic metabolism. Purple nonsulfur bacteria will not, for example, grow on carbon compounds such as butanol that are more reduced than cell biomass, unless they are grown under nitrogen-fixing conditions to allow H_2 to be produced as a way of relieving excess reducing equivalents (60). Many fermentative bacteria can produce more ATP if they are permitted to use protons as an electron acceptor to generate H_2. This has the effect of increasing the amount of ATP available for cells to use to fix nitrogen. Another potentially useful product is CH_4, which is produced by cells of *R. palustris*, *R. rubrum*, *R. capsulatus*, and *A. vinelandii* when they are grown under nitrogen-fixing conditions using Fe-only nitrogenase. The quantities of CH_4 produced are small, but they are sufficient to support the growth of an obligate CH_4-utilizing *Methylomonas* strain in coculture (109) (**Figure 6**).

CONCLUSIONS

The idea that alternative nitrogenases are fail-safe enzymes that can substitute for Mo nitrogenase in supporting global biological nitrogen fixation is supported by their widespread occurrence in bacteria and archaea and by increasing evidence that they are expressed and active in nature. The term fail-safe should not be taken to mean unimportant. It is increasingly being recognized that V and Fe-only nitrogenases may play critical roles in nitrogen fixation in some ecosystems. In addition, these alternative nitrogenases probably play ancillary roles in supporting microbes and microbial communities that depend on H_2 as a redox relief mechanism and CH_4 as a carbon source.

SUMMARY POINTS

1. There are three forms of nitrogenase: Mo nitrogenase, V nitrogenase, and Fe-only nitrogenase.
2. Mo nitrogenase evolved first and is the most efficient of the three enzymes in converting N_2 to NH_3.
3. The alternative V and Fe-only nitrogenases are considered to be backup enzymes, used by bacteria and archaea when Mo is limiting.
4. V and Fe-only nitrogenases have VFe and FeFe cofactors, instead of a MoFe cofactor, at their active sites.
5. The two alternative nitrogenases are found only in microbes that also have Mo nitrogenase.
6. V and Fe-only nitrogenases are active in the environment, and V nitrogenase may contribute to global biological nitrogen fixation in terrestrial regions of the subarctic.
7. Fe-only nitrogenase produces significant quantities of CH_4, and this may support growth of microbial communities in nature.
8. All three nitrogenases produce considerable quantities of H_2.

FUTURE ISSUES

1. Almost nothing is known about the physiology and regulation of V and Fe-only nitrogenases in methanogenic archaea or in the firmicutes, especially members of the *Clostridia*.
2. Very little is known about the molecular mechanisms regulating the expression of alternative nitrogenases, especially in bacteria that do not regulate gene expression by molybdate repression.
3. The full range of small molecules that can be reduced by alternative nitrogenases is not known.
4. There are between 5 and 20 genes that are coordinately regulated with V nitrogenase and Fe-only nitrogenase structural genes, but their functions are mostly unknown.
5. The contributions of V and Fe-only nitrogenases to the global nitrogen cycle have yet to be systematically explored.
6. Microbes that express Fe-only nitrogenase may play a role in shaping microbial community interactions through production of CH_4 as well as NH_3. But this has yet to be explored.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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