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Review Article

Survey on nitrogenase evolution by considering the importance of nitrogenase, its structure, and mechanism of nitrogenase

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Abstract

Nitrogenase is a complicated enzyme that actives the ATP-dependent reduction of dinitrogen (N_2) to ammonia (NH_3). The aim of this manuscript is to review the nitrogenase evolution with considering nitrogenase, structure of nitrogenase, action mechanism of nitrogenase and oxygen sensitive mechanism of nitrogenase. The searches focused on publications from 1980 to February 2023, using PubMed, Google Scholar, Science Direct, and Scopus databases. In the term of evolution, the nitrogen cycle has experienced highly changes; at the beginning of life and suggested the exact anoxic scenario, the comparatively sufficient ammonium was possibly used in an assimilation/mineralization cycle by protocellular organisms. The main nif gene products which are active in nitrogen fixation are nifH, nifD, nifK, nifT, nifY/nafY, nifE, nifN, nifX, nifU, nifS, nifV, nifW, nifZ, nifM, nifF, nifL, nifA, nifB, fdxN, nifQ, and nifJ. The main vnf gene products which are active in nitrogen fixation are vnfA, vnfE, vnfN, vnfX, vnfH, vnfFd, vnfD, vnfG, vnfK, and vnfY. Oxygen can be either detrimental or beneficial for diazotrophs in organisms suitable for an aerobic catabolism, and it supports the production of a substrate for nitrogenase (ATP), but it can also impede the activity and suppress the synthesis of this enzyme.

Keywords: biological nitrogen fixation; nitrogenase; nitrogenase evolution; oxygen; structure of nitrogenase

Introduction

N_2 fixation takes place in three distinct ways: (i) through geochemical procedures such as lightning, (ii) biologically via the action of the enzyme, nitrogenase found just in a select group of microorganisms, and (iii) industrially by the Haber-Bosch process (Hoffman *et al*., 2014). Nitrogenase catalyzes the ATP-dependent reduction of dinitrogen to ammonia, which is basic to the proceeding of biological nitrogen fixation. The nitrogenase complex contains two Fe protein dimers and one MoFe protein tetramer; each constituents harbours metalloclusters that interfere in electron flow to the active centre within the complex, the active centre of Mo-nitrogenase, formed the FeMoco or the M-cluster is a [$MoFe_7S_9C$-homocitrate] cluster; electrons are transferred from the [Fe_4S_4] cluster in the Fe protein to the P-cluster and then the M-cluster in the MoFe protein, where substrate reduction happens; nitrogenase catalyses the ATP dependent reduction of dinitrogen

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(N_2) to ammonia (NH_3) under ambient temperature and pressure, and nitrogenase has been organized to catalyse the reduction of alternative substrates, including H^+, N_3^-, CN^-, C_2H_2 and CO (Smith, 1977; Lery *et al*., 2010). The Mo-, V- and Fe-only nitrogenases are all two-component enzyme systems where the catalytic element (dinitrogenase) is known as either the MoFe, VFe, and FeFe protein and the component activated in electron delivery (dinitrogenase reductase) in the Fe protein (Burgess and Lowe, 1996; Eady and Prevent, 1996; Hu *et al*., 2012). The Mo-, V-, and Fe-only nitrogenases are mechanistically alike, but genetically distinct as they are encoded by different gene clusters designated, nif, vnf, and anf, respectively (Eady and Prevent, 1996; Harris *et al*., 2017). Traditional Haber-Bosch procedure for producing NH_3 from N_2 is a high energy-consumption process with nearly 1.9 metric tons of fossil CO_2 being released per metric ton of NH_3 produced, and catalytic NH_3 synthesis is an attractive and promising alternative approach (Yang *et al*., 2019). Fundamental advances in speculating nitrogenase structure and function during those intervening years included the following: (i) it was designed that nitrogenase is a two-component system composed of the MoFe protein (also called dinitrogenase or component I), and reductase or component II. (ii) A reducing source and MgATP are required for catalysis. (iii) Fe protein and MoFe protein associate and dissociate in a catalytic cycle consisting single electron transfer and MgATP hydrolysis. (iv) It was found that the MoFe protein consists of two metal clusters: the iron-molybdenum cofactor (FeMo-co), which provides the active site for substrate binding and reduction, and P-cluster, involved in electron transfer from the Fe protein to FeMo-co. (v) Crystallographic structures were solved for both Fe and MoFe proteins. (vi) Also, the substitute V- and Fe-type nitrogenase, in which the Mo of FeMo-co is replaced by V or Fe, was discovered (Hoffman *et al*., 2014). The aim of this article is to survey nitrogenase evolution by considering the importance of nitrogenase.

Nitrogenase evolution

Legumes nodules are cylindrical or spherical growths shaped on the plant roots because of an infection by bacteria of the genera *Azorhizobium*, *Rhizobium*, or *Bradyrhizobium* (Yong *et al*., 2018; Ogbaji *et al*., 2018). These infections are called mutualistic for the reason that they lead to advantages to both partners, and the bacteria which increase in tremendously specialized cells in the center of the nodule, conduct a reaction of fundamental biological usefulness, namely nitrogen fixation (Seefeldt *et al*., 2013; Shahrajabian *et al*., 2019a, b):

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

Nitrogenase is a complicated metalloenzyme that activate a principal step in the global nitrogen cycle-the reduction of inert dinitrogen (N_2) to bioavailable ammonia (NH_3), and three homologous nitrogenase have been recognized, which resemble one another in primary sequence and cluster type: -Molybdenum (Mo), Iron (Fe)-, and Vanadium (V)- only nitrogenase, and all of them are two-component systems containing of: Fe protein, VFe, MoFe, and FeFe protein, and Mo-nitrogenase shows absolutely different biochemical characteristics containing of different protein composition, low requirement of Mg.ATP, insensitivity towards O_2, and a Mo-co type of cofactor at the active site (Gerloff *et al*., 1993; Shayanfar *et al*., 2011; Esfandiary *et al*., 2011). Nodules considered as organs of gas exchange, and the outer cell layers of the nodule are more relatively organized than those of the root, and the nodule contains of two general tissue regions, an outer layer of cortex and the central infected region (Shahrajabian *et al*., 2022). The cortex can be separated into a loosely organized outer layer of cells and a compact inner layer (Webb and Sheehy, 1991). Biological nitrogen fixation (BNF) can only happen in a severe anaerobic environment, because the nitrogenase (molybdenum ferritin and ferritin) is greatly sensitive to oxygen, (Khoshkharam *et al*., 2021), and once subjected to oxygen, nitrogenase deactivates rapidly, therefore these NFMs developed multiple mechanisms to determine this contradiction, though, (i) extraordinary respiration consuming the formed oxygen and making enzyme in a low-oxygen ambient, (ii)

combination oxygen by leghemoglobin, and (iii) established a mucosal layer preventing oxygen passing through (Tian *et al*., 2019). Nitrogen-fixing and phosphorus solubilizing cyanobacteria play a dominant function in nitrogen mobilization and phosphorus solubilization for the advantages of plant growth (Sun *et al*., 2021), and the cyanobacterial species-rich in biological soil crust, which is important for fixing atmospheric nitrogen in the soil, soil adherence, retain moisture, and preventing erosion by extracellular polymorphic substances (Xie *et al*., 2007; Vinoth *et al*., 2020). Photosynthetic bacteria are famous for their proportional broad substrate application in the light, being especially useful at growing on organic acids (Ogbaji *et al*., 2013; Soleymani *et al*., 2013; Liu et al., 2015; Soleymani *et al*., 2016). It has been noted that that nodulated rhizobia may prompt plant growth on metal contaminated sites directly by phosphate solubilization, nitrogen fixation, or siderophore formation (Neubauer *et al*., 2000; Mar *et al*., 2011; Karthik *et al*., 2017), moreover, some rhizobial exudates can also immobilize metal ions, thus decreasing their negative impacts on plants (Neubauer *et al*., 2000; Esfandiary *et al*., 2012; Shahri *et al*., 2012; Siegbahn, 2019). *Bradyrhizobium* strains have been shown as rhizobial symbionts of only a minority of Lotus species, albeit these papers do not refer to plants growing in heavy metal polluted areas (Khoshkharam *et al*., 2010; Shahrajabian *et al*., 2011; Soleymani *et al*., 2011 a,b; Lorite *et al*., 2012; Lorite *et al*., 2018).

The evolutionary process for the development of nitrogenase arises from the ever-expanding biosphere and the constant state production of decreased nitrogen from abiotic sources (Mus *et al*., 2018). The great part of the reduced abiotic nitrogen in the Archean oceans was achieved from lighting and volcanic origins on the order of 10^{10}-10^{1}1 mol of N per year (Som *et al*., 2016). In the reducing environment of Archean oceans, Mo-availability was reduced in comparison to modern levels, as it was seized in sedimentary sulfides (Erickson and Helz, 2000; Wang, 2012). The decreasing condition of the Archean Sea leads to limited Mo bioavailability and high Fe bioavailability for the Archean life (Mus *et al*., 2018). Because of these situations, a logical theory has been announced that the substitute nitrogenases preceded the Mo-nitrogenase in the evolutionary lineage (Anbar and Knoll, 2002; Zhang *et al*., 2014). The transfer of nitrogenase genes from archaea to the bacteria most probably happened between an anaerobic firmicute and a methanogen which would be in concurrence in the environment (Stams, 1994; Boyd *et al*., 2011). Earlier phylogenetic studies supported the concept that anf and vnf lead to the dispersal and evolution of the more effective nif, via gene duplication and gene transfer (Raymond *et al*., 2004).

The evolution of nitrogenase is a joined shaping of the gene's operon, protein structure, and maturation of the metal cofactors (Mus *et al*., 2018). A theory proposed that proto-nitrogenase could have offered a role as a detoxyase because of the lack of evolutionary pressure for fixed biotic N_2 before the nitrogen crisis (Navarro-Gonzalez *et al*., 2001). This would show that the development of the proto-metal active sites in the enzyme was largely as a general reductase (McGlynn *et al*., 2012). Nitrogenase and NflD share akin structural active sites, and probably, the common ancestors were appropriate for binding similar porphyrins or metal clusters (Boys *et al*., 2011; McGlynn *et al*., 2012). During an oxidoreductase reaction, these metal clusters could have started as simple Fe-S clusters that acted as the electron donor (Mus *et al*., 2018). The natural selection for the most effective and certain nitrogenase involves the fine-tuning of its metal cofactors (Mus *et al*., 2018). NifB makes the early precursor of the FeMo-cofactor known as NifB-co which has been suggested as the Fe6-S9-X core of the FeMo-cofactor (Curatti *et al*., 2005; George *et al*., 2008); without the scaffolding genes of nifEN the FeMo-cofactor is not capable to link with nifDK, and there is a build-up of the NifB-co (Tal *et al*., 1991; Shah *et al*., 1994). The NifB-co has been proved to be active in the apo-nifDK enzyme and capable of decreasing acetylene and hydrogen but not nitrogen (Soboh *et al*., 2010). NifEN with the FeMo-cofactor has also been shown to be catalytically active but at a much lower rate (Hu *et al*., 2009). The operon duplication of nifDK to nifEN was important for the maturation of the FeMo-cofactor due to the high selection influence of a more effectual nitrogen fixer (Mus *et al*., 2018). The operon duplication events for the substitute nitrogenase lead to the evolution of differentiated metal loading in the cofactor because of the environmental pressure of metal

availability (Mus *et al*., 2018). The co-evolution of the nifENB with the nitrogenase enzyme reinstates the condition the nif operon being the source of the possible nitrogenase (Mus *et al*., 2018).

In the term of evolution, the nitrogen cycle has experienced highly changes. At beginning of life and offered an accurate anoxic scenario, the comparatively sufficient ammonium was probably utilized in an assimilation/mineralization cycle by protocellular organisms. To replenish ammonium pool, a vast N_2 reservoir obtaining from the primitive protosolar nebula was chemically decreased to ammonium at hydrothermal vents by FeS/H_2S of abiotic origin. Simultaneously, N_2 was also oxidized by atmospheric lightning to nitric nitrogen, which was applied as ammonium source via nitrite respiration by Nrf and/or nitrite and NO detoxification by incomplete denitrification (ancient Nar, Nir, and Nor). To detoxify hydroxylamine, a highly toxic, dead-end by-product of nitrite reduction to ammonium, hydroxylamine reductase (Hcp) could be acquired, along with anammox through Hzh coupled to NirS and Hzo, which recycles factor of the fixed nitrogen back to N_2. Appearance of assimilatory Nas and Nir was also subject to significant evolutionary pressure to replenish ammonium pool. Nitrate reductases are allocated in three clades: the assimilatory eukaryotic cytosolic enzyme and the prokaryotic enzymes Nas/Nap, and Nar.

Although, the eukaryotic enzyme seems to have a monophyletic source, Nap and Nar were possibly obtained by horizontal gene transfer, Nap being a subclade of Nas.

Eukaryotic assimilatory Nir is a chloroplastic siroheme enzyme, considering bacterial Nir may include diverse cofactors and are either NO-producing periplasmic enzymes (NirS and NirK) or NH_4^+ -producing membrane-bound (NrfA) or cytosolic (NirB) enzymes. Another stage could be added to the biological nitrogen cycle when the nitrogenase systems started to catalyze a six-electron transfer to N_2 to yield ammonium, therefore contributing to restore ammonium reservoirs. This system could be completed from a primitive Fe nitrogenase to the actual MoFe or VFe enzymes. The procedure possibly included duplication of former gene pairs to yield the present nifDK and nifEN operons. Even though, the extensive distribution of nif genes among archaea and bacteria archaea proposes an advanced appearance of N_2 fixation, this could have been because of horizontal gene transfer. The complexity and energetic cost of the process and the lack of nif genes in eukaryotic organelles fully support a late emergence of biological N_2 fixation as a dominant step of the nitrogen cycle.

Two billion years ago, contributing of oxygenic photosynthesis greatly altered the whole earth's ecosystem for an acute transition from anoxic to oxic environments. Oxygen appearance made practicable bioavailability of copper, sequestered as sulfidic minerals, and evolution of high-yielding aerobic respiration according to the heme-copper oxidases. Appearance of the Cu enzymes NirK and Nos permitted complete denitrification and closed the nitrogen cycle by producing N_2. Explosion of aerobic life shapes reinforced the initial primal of nitrate assimilation to ammonium, and the increasing O_2 pressure made preferable the emergence of aerobic nitrification via Amo, and Hao, as a consequence adding the final step to the extant nitrogen cycle. In conclusion, evolution of the biological nitrogen cycle could be anticipated from a noncyclic route along with an incomplete denitrification step that was provided with nitrogen from immeasurable reservoirs of N_2 and nitrate and that evolved to manufacture ammonium to comply with nitrogen demands of protocells. Recycling of ammonium was first achieved by ammonium assimilation/mineralization, nitrite respiration, nitric nitrogen assimilation, and thereafter by N_2 fixation, though N_2 was mostly recycled through anammox. Some procedures also evolve for detoxifying plans, such as hydroxylamine or NO handling enzymes. Transition from anoxic to oxic environment and copper bioavailability causes closing of the nitrogen cycle by complete N_2-producing denitrification and oxygen-dependent nitrification. In fact, more than half of the nitrogen fixed that comes into the ecosystems has anthropogenic root. Human activities are changing very rapidly the amounts of nitrogenous constituents at the level of all nitrogen reservoirs and thus dynamics of these compounds and constituents of bacterial and archaeal populations in terrestrial, freshwater, and marine environments. As the composition of these complex ecosystems and their dynamics are not discovered precisely, it is not probable to make precise predictions about evolution of the nitrogen cycle in the long time, but it is necessary to include human being as one of the most principal parameters influencing the nitrogen equilibrium in the biosphere at a global scale in the next future. From the first studies of nitrogenase, it was

found that ATP hydrolysis is belonging to delivery of electrons and reduction of N_2, yet the order of ATP hydrolysis and electron transfer, which regulates the nature of the coupling, was never established, and the new discoveries permit completion of the thermodynamic cycle undergone by the Fe protein, indicating that the energy of ATP binding and protein-protein association drive electron transfer (ET), with subsequent ATP hydrolysis and Pi release leading to dissociation of the complex between the $Fe^{ox}(ADP)_2$ protein and the reduced MoFe protein (Duval *et al*., 2013).

Nitrogenase

This extremely complex enzyme actually includes two proteins: an iron rich protein (Fe protein) and a protein consisting both molybdenum and iron at the active site (MoFe protein) (Orme-Johnson, 1985; Dean *et al*., 1993). Biological nitrogen fixation is the conversion of atmospheric nitrogen into ammonia ($N_2+6H^++6e^-=2NH_3$), and is activated by nitrogenase, containing the MoFe and Fe proteins (Johnson *et al*., 2000). Three distinct kinds of nitrogenase have been proved to exist in diazotrophs: the Mo-nitrogenase, the V-nitrogenase, and the Fe-only nitrogense (Hartmann and Barnum, 2010). Mo-nitrogenase is a complicated two component metalloenzyme constituted of two proteins, the smaller dimeric component, calls as the iron protein or dinitrogenase reductase (NifH protein), actives as an ATP-dependent electron donor to the larger heterotetrameric component, indicates as the molybdenum-iron protein or dinistrogenase (comprising the NifD and NifK component proteins), which consists of the enzyme catalytic site (Mus *et al*., 2018). Both of the nitrogenase-component proteins are highly oxygen sensitive, the two other nitrogenase, V-nitrogenase and Fe-only nitrogenase, called as alternative nitrogenases, are enzyme homologs, with the exception of an additional subunit (VnfG or AnfG) in the dinitrogenase constituent, and the absence of the heteroatom Mo (Coucouvanis *et al*., 1996; Mus *et al*., 2018).

Mo-nitrogenases and [FeFe]-hydrogenases are two groups of complex Fe-S cluster containing enzymes that activate fundamental procedures on Earth, even though these enzymes are not evolutionarily connected, various unifying characteristics that have been newly uncovered by comparing the biosynthesis of their complex Fe-S clusters may show that these systems followed indistinguishable evolutionary paths, at least in the context of how cluster modifications that define their extraordinary catalytic possessions are introduced (Shepard *et al*., 2011). The structural constituents of the Mo-nitrogenase are encoded by nifh, nifD, and nifK genes, and the V-nitrogenase is encoded by vnfH, vnfD, vnfG, and vnfK, and the Fe-only nitrogenase ingredients are products of anfH, anfD, anfG, and anfK genes (Sellmann *et al*., 2000; Mus *et al*., 2018). The most considerable common characteristic linking the nitrogenase FeMo-co to the 2Fe subcluster of the H-cluster are the minimal coordination by protein ligands, when compared to other Fe-S enzymes, and the application of peculiar non-protein lignads (carbon, monoxide, cyanide, homocitrate) in their place (Shepard *et al*., 2011). The sophisticated complexity of FeMo-co and H-cluster biosynthesis is unquestionably the result of a high degree of evolutionary refinement (Shepard *et al*., 2011). A complicated metallo-organic species called FeMo-cofactor gives the site of substrate reduction within the MoFe protein, but precisely where and how substrates interrelate with FeMo-cofactor remains unrevealed, and on the basis of latest findings, the MoFe protein α-70^{Val} residue, whose side chain approaches one Fe-S face of FeMo-cofactor, plays a significant function in defining substrate access to the active site (Dos Santos *et al*., 2007).

Site of substrate reduction is nitrogenase molybdenum-iron (MoFe) protein, also defined as dinitrogenase; the obligate electron donor to MoFe protein is nitrogease iron protein (Fe protein), also known as dinitrogense reductase (Halbleib and Ludden, 2000); MoFe protein is consisted of two various polypeptides encoded by nifD and nifK genes; the Fe protein is encoded by the nifh gene (Igarashi and Seefeldt, 2003; Hoffman *et al*., 2009; Shahrajabian *et al*., 2021a,b). The Fe protein also consists of sulfur, organized into Fe_4S_4 clusters (Riaziat *et al*., 2012; Yazdpour *et al*., 2012); it is an electron transfer protein, providing single electrons

to the MoFe protein at a cost of 2 ATP/e^-. The MoFe protein is about four times larger than the Fe protein, is the site of all substrate reductions, and includes 30 Mo and 2 Fe organized in metalloclusters at the active site (Streeter, 1995). Both proteins require and operate in a highly reducing situation and are highly sensitive to molecular oxygen, having a half-life of < 10 min in air (Eady and Postgate, 1974). Another substrate for nitrogenase is protons; in fact, the reduction of H^+ is obligatory; the formation of one H_2 must go along with the reduction of every N_2, so the allocation of electrons is about 25% to H^+ and 75% to N_2 under normal conditions (Simpson and Burris, 1984).

Nitrogenase is remarkable for photosynthetic hydrogen generation with purple non-sulfur bacteria (PNSB), and the activity of nitrogenase is positively connected with hydrogen production performance of PNSB (Wang *et al*., 2010; Yang *et al*., 2012; Sun and Shahrajabian, 2023; Sun *et al*., 2023), and the disruption of hupSL genes resulted in increased of nitrogenase expression level and nitrogenase activity (Wang *et al*., 2014), consequently, boosting nitrogenase expression level could enhance the hydrogen production performance. Yang *et al*. (2015) suggested that expressing nitrogenase related genes could be a substitute way for increasing the hydrogen production performance of purple non-sulfur bacteria. It was also proposed that as FeMo-cofactor needs the four electrons required for optimal binding of N_2, each successive pair of electrons is stored as an Fe-H^--Fe bridging hybride, with the FeMo-cofactor metal-ion core retaining its resting redox state (Seefeldt et al., 2012). Streeter (1995) proposed a new model for the rapid impacts of non-invasive treatments on nitrogenase and respiratory activity in legume nodules, and consequences of argon, acetylene, and oxygen may be because of disturbance of a delicate balance in the supply and demand for protons in bacteroids and/or a disturbance of the balance between the consumption of oxygen in mitochondria and bacteroids. Sujkowska-Rybkowska *et al*. (2020) showed that in the heavy metal-contaminated soils *Lotus corniculatus*, and *Anthyllis vulneraria* are influenced by *Bradyrhizobium* strains harboring symbiotic *nifD* gene haplotypes phylogenetically unconnected to Clade II strains that predominate in Europe among bradyrhizobia nodulating *Genisteae* and *Loteae* spp. In legumes grown under symbiotic conditions, drought brings about a rapid inhibition of symbiotic nitrogen fixation, an inhibition that has been shown to happen before this of photosynthetic activity (Djekoun and Planchon, 1991; Aldasoro *et al*., 2019). Nitrogen-fixing cyanobacteria contains a central position in the nutrient cycling largely because of their inherent capacity to fix atmospheric nitrogen directly into ammonium, with the assist of the enzyme nitrogenase; thus, making it accessible for use by higher plants (Kumar and Kumar, 1988; Shahrajabian *et al*., 2020 a). Cyanobacteria possess nitrate and nitrite reductase which convert nitrate to nitrite and nitrite to ammonia (Guerrero *et al*., 1981). Nitrogen reductase of cyanobacteria is a membrane bound enzyme and utilizes reduced ferredoxin rather than a reduced pyridine nucleotide as its electron donor (Guerrero *et al*., 1981).

In cyanobacteria, treatment with low concentrations of $NaHSO_3$ can increase photosynthetic efficiency, therefore $NaHSO_3$ in high amounts often hinders cell growth and photosynthesis may even cause death; the impacts of treatment with low, moderate, and high concentrations of $NaHSO_3$ in cyanobacteria were proposed (Wang *et al*., 2010). It has been reported that ultraviolet-B irradiance has differential impacts on the enzymes of the nitrogen metabolism in the cyanobacterium *Nostoc calcicola*, and N_2-fixing cyanobacteria are being considered as a substitute natural source of nitrogenous fertilizers for rice and other crops (Kumar *et al*., 1996). Oxygen interferes with biological nitrogen fixation at various levels, and at genetic level, oxygen represses the nitrogenase synthesis (Akkose *et al*., 2009), and at enzyme activity level, oxygen causes a unpredictable inhibition (switch-off) of nitrogenase activity in *R. sphaeroides* (Goldberg *et al*., 1987). It has been reported that the biochar (BC) and pelelith (PL) additions boosted the sensitivity of N-fixing bacteria to environmental changes, and fluorescein diacetate hydrolase (FDA), TN, moisture content, and NO_3-N significantly influenced N-fixing bacteria at genus level (Jiang *et al*., 2021). It has been proposed that there are different techniques observed in cyanobacteria (spatial and temporal separation) to increase N_2 fixation were connected with small changes in the nif nucleotide sequences, and the progress of the N_2 fixation procedure will be directly

related to H_2 production as one of the leading contenders for renewable energy (Balzer *et al*., 1995; Esteves-Ferreira *et al*., 2017).

Structure of nitrogenase

Nitrogenase is a complicated, bacterial enzyme that actives the ATP-dependent reduction of dinitrogen (N_2) to ammonia (NH_3), and it includes of two proteins, the catalytic molybdenum-iron protein (MoFeP), and its specific reductase, the iron protein (FeP) (Sarma *et al*., 2010). A defining characteristic of nitrogenase is that electron and proton transfer processes associated with substrate reduction are synchronized by conformational alternates driven by ATP-dependent FeP-MoFeP interactions (Bolin *et al*., 1991; Hales and Oliver, 1991; Kang *et al*., 2020). Nitrogenase composed of two protein constituents, the homodimeric Fe-protein, the structural subunits of which are encoded by the *nifH* gene, and the heterotetrameric MoFe-protein encoded by the structural genes *nifK*, and *nifD*, and metalloclusters are related with both components (Peters and Szilagyi, 2006). Some other N_2-fixing organisms contains a more restricted nif gene set, for example *Frankia* sp. contains 12 nif genes (nifH, nifD, nifK, nifE, nifN, nifX, nifW, nifZ, nifB, nifU, nifS, nifN) (Oh *et al*., 2012), *R. meliloti*, *B. japonicum*, and *A. caulinodans* include at least 9 nif genes (nifH, nifD, nifK, nifE, nifN, nifB, nifS, nifW, nifX, nifA) (Fischer, 1994), and *M. maripaludis* and *Paenibacillus* sp. consist of 9 nif genes (nifB, nifH, nifD, nifK, nifE, nifN, nifXhesA, nifV) (Wang *et al*., 2013). MgATP is completely needed for electron transfer from the $[4Fe\text{-}4S]^0$ Fe protein to the reduced state of the MoFe protein, and in that reaction both electrons are transferred and are applied to reduce substrate (Angove *et al*., 1998). The most frequently occurring nitrogenases have Mo in their active center, the iron-molybdenum cofactor; in addition to Mo-nitrogenase, some species contain homologous alternative nitrogenases, namely, a vanadium-containing nitrogenase and/or the so-called iron-only nitrogenase (Sippel and Einsle, 2017; Bergmann *et al*., 2021).

MoFe, VFe, and FeFe nitrogenases catalyze the reduction of the inert nitrogen to the metabolically functional ammonia (Zanello, 2019). Differential gene expression shows *anf* is more rigorously controlled than *vnf*, and charge variations at the G subunit of AnfDGK suppress interactions with NifH and VnfH (Pence *et al*., 2021). Nitrogenase is extremely sensitive to N_2 and O_2 fixation is energetically expensive, "costing" a cell up to 16 molecules of ATP per molecules N_2 reduced (Bergmann *et al*., 2021). The activity of the principal enzyme, nitrogenase, is controlled by reversible mono-ADP-ribosylation of one of its constituents, the Fe protein, and this protein supplies the other component, the MoFe protein, with the electrons needed for the reduction of molecular nitrogen (Nordlund and Hogbom, 2013). The Fe-protein is ADP-ribosylated and de-ADP-ribosylated by dinitrogenase reductase ADP-ribosyl transferase and dinitrogenase reductase activating glycohydrolase, respectively (Nordlund and Hogbom, 2013). Nitrogenase enzymes are countless but biochemically similar and so the entire family of enzymes is related to as "nitrogenase" even though the name does not conform to systematic enzyme nomenclature (Marchal and Vanderleyden, 2000; Einsel and Rees, 2020). The enzyme complex nitrogenase includes of two proteins, a molybdenum iron protein (Protein 1) that binds N_2 (dinitrogenase reductase), and a smaller iron protein (Protein 2) that acts as an electron donor to Protein 1 (nitrogenase reductase), although substitute nitrogenases that include vanadium or only iron in the larger subunit have been recognized (Peters *et al*., 1995). Like catalyzing the reduction of N_2 to NH_3, nitrogenase can decrease other small triply bonded molecules including acetylene, azide, and cyanide, and nitrogenase reduces hydrogen ions to gaseous hydrogen, even when N_2 is present (Wang and DeBeer, 2021). The [MoFe] nitrogenase system includes a homodimeric reductase constituent (Fe protein), and a tetrameric catalytic component (MoFe protein), and upon association of the two ingredients, an electron is transferred from the Fe protein's [4Fe-4S] cluster to the MoFe protein's M-cluster through the intermediate MoFe protein P-cluster; the cofactors which promote this electron transfer include the cubane-type [Fe-S] cluster, P-cluster

[Fe_8S_7], and M-cluster [homocitrate: $MoFe_7S_9C$]. Within the catalytic MoFe protein, the M-cluster is ligated by an organic homocitrate and two residues: C275 and H442. Structural homologs to the M-cluster consist of the native V-cluster [VFe_7S_9C] (substitution of molybdenum with vanadium) and symmetric L-cluster [Fe_8S_9C] (Georgiadis *et al*., 1992; Barbosa *et al*., 2002; Huang *et al*., 2021). In all nitrogenase systems, Fe proteins couple the transfer of an electron achieved from either a ferredoxin or a flavodoxin to their cognate dinitrogenase (MFe protein, with M = Mo, V or Fe) to the hydrolysis of two molecules of ATP per electron; three dimensional structure of the complex of the two constituents of Mo-nitrogenase, Fe protein NifH, and the MoFe protein NifDK, and the complex is shaped transiently for each single electron transfer and the overall reaction demands at least 8 electrons per N_2 (Trncik *et al*., 2022). Comparison of the particular activities for N_2, H_2, and C_2H_2 for the Mo-, V-, and Fe-only nitrogenases isolated from *A. vinelandii*, indicated that the specific activity for N_2 in Mo-Nitrogenase is 605 nmol min^{-1} mg^{-1}, specific activity for H_2 for Mo-Nitrogenase is 2226 nmol min^{-1} mg^{-1}, and certain activity for C_2H_2 in Mo-Nitrogenase is 1819 nmol min^{-1} mg^{-1} (Harris *et al*., 2017); specific activity for N_2, definite activity for H_2, and specific activity for C_2H_2 in V-Nitrogenase is 660 nmol min^{-1} mg^{-1}, 1400 nmol min^{-1} mg^{-1}, and 220 nmol min^{-1} mg^{-1} (Sickerman *et al*., 2017). Specific activity for N_2 in Fe-only nitrogenase is 181 nmol min^{-1} mg^{-1}, while particular activity for H_2, and definite activity for C_2H_2 for Fe-only nitrogenase are 1085 nmol min^{-1} mg^{-1}, and 306 nmol min^{-1} mg^{-1}, respectively (Harris *et al*., 2017). X-ray spectroscopy has played a dominant function in the understanding of nitrogenase cofactors for more than three decades (Kowalska and DeBeer, 2015). NifS and NifU provide the basic conversion of cysteinyl thiolates and iron incorporation to produce the two inorganic Fe_4S_4 iron-sulfur clusters to be transferred onto NifB for carbide insertion (Joseph *et al*., 2020). The nifD and nifH promoters in cowpea Rhizobium IRc78 have a hexameric (G-G-T-T-G-C) and a pentameric (T-G-G-C-A) sequence in common (Yun and Szalay, 1984). Seefeldt *et al*. (2009) reported that all substrates for nitrogenase appear to bind o a single FeS face of FeMo-cofactor requested by the MoFe protein amino acid α-70^{Val}, and the binding of different substrates to the same site within FeMo-cofactor, but at different redox states, can explain the non-reciprocity with regarding to their competition for occupancy of the active site, a combination of amino acid substitution and expeditious freezing has proven prospering for trapping a number of alkyne and nitrogenous substrates on FeMo-cofactor, and the state trapped during reduction of the alkyne substrate propargyl alcohol consists of a reduced form of the substrate bound to FeMo-cofactor. ^{13}C- and $^{1/2}H$-ENDOR spectroscopy indicate that this is the allyl-alcohol ($HO\text{-}CH_2\text{-}CH{=}CH_2$) reduction product bound side-on to one Fe4 (Seefeldt *et al*., 2009). The P-cluster in the MoFe protein functions in nitrogenase catalysis as an intermediate electron carrier between the external electron donor, the Fe protein, and the FeMo-co sites of the MoFe protein, and it has been recognized that the three oxidation states differ in coordination, implicating that the P^{1+} state retains the serine hydroxyl coordination but absence of the backbone amide coordination observed in the P^{2+} states (Keable *et al*., 2018). A molecular mechanism for the translocation of NH_3 by skipping via a sequence of hydrogen bonds involving eleven water molecules and surrounding amino acids has been progressed in the three species *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Clostridium pasteurianum*, and the putative mechanism needed movement aside of some water molecules by up to ~ 1A°, and the surrounding protein is comprised of various chains and has little auxiliary structure protein fluctuations are part of the mechanism (Dance, 2013). Electron transfer (ET) from the Fe protein to the catalytic MoFe protein consists of a series of synchronized events needing the transient association of one Fe protein with each $\alpha\beta$ half of the $\alpha_2\beta_2$ MoFe protein, and this procedures is related to as the Fe protein cycle and consists of binding of two ATP to an Fe protein, association of an Fe protein with the MoFe protein, ET from the Fe protein to the MoFe protein, hydrolysis of the two ATP to two ADP and two P_i for each ET, P_i release, and dissociation of the oxidized Fe protein-$(ADP)_2$ from the MoFe protein, because the MoFe protein tetramer has two separate $\alpha\beta$ active units, it participates in two distinct Fe protein cycles, and a practicable mechanism for communication between the two halves of the nitrogenase complex is proposed by normal-mode calculations indicating correlated and anti-correlated

motions between the two halves (Pierik *et al*., 1993; Hoffman *et al*., 2009; Lancaster *et al*., 2011; Kowalska *et al*., 2015; Danyal *et al*., 2016; Bjornsson *et al*., 2017).

Li *et al*. (2022) demonstrated that the possibility of Fe-Mo base as an electrocatalyst for nitrogen reduction reaction (NRR) and laid the track for the creation of a nitrogenase-like biomimetic electrocatalysts to gain the effective N_2 fixation. nif genes have been recognized in 21 out of 44 sequences cyanobacteria genomes thus far, consisting of terrestrial and marine strains (Boyd and Peters, 2013). They are organized in separate operons namely nifB-fdxN-nifSU, nifHDK, nifENXW, and nifVZT (Esteves-Ferreira *et al*., 2017). The intrinsic capability for N_2 fixation in cyanobacteria is connected only to the nitrogenase enzyme system, with the molybdenum nitrogenase (Mo-nitrogenase) being the most studied nitrogenase (Betancourt *et al*., 2008; Jasniewski *et al*., 2018).

There are three kinds of dinitrogenases usually found in cyanobacterial nitrogenases, which vary connecting with the metal content. Thus, type 1 contains molybdenum (Mo), type 2 contains vanadium (V), and type 3 iron (Fe). The reduction of nitrogen (N_2) to ammonia (NH_3) requires metabolic energy in the form of ATP, and in this context two ATP molecules are used for each electron transferred from the dinitrogenase reductase to dinitrogenase. Therefore, the reaction needs a total of 16 ATP molecules until the dinitrogenase has accumulated enough electrons to decrease N_2 to NH_3. In addition, this reaction goes with the reduction of two protons (H^+) to one hydrogen molecules (H_2) (Esteves-Ferreira *et al*., 2017). Nif E, and NifN is a hetero-tetrameric complex look like to NifD and NifK respectively, and seems to act as scaffolds for FeMo-co assembly (Fani *et al*., 2000). NifV is a homocitrate synthase which provides homocitrate for the FeMo-con biosynthesis (Zheng *et al*., 1997; Mayer *et al*., 2002). NifS and NifU are involved in iron and sulphur mobilization, and in the assembly of [4Fe-4S] clusters (Rubio and Ludden, 2008). Therefore, these [4Fe-4S] clusters are transferred to NifB, and converted into NifB-cofactor, a precursor for the biosynthesis of FeMo-co (Curatti *et al*., 2007). NifX is capable of binding precursors of the FeMo-co, binding a transient reservoir for these molecules (Hernandez *et al*., 2007). NifW is not directly active in the FeMo-protein assembly, but correlated with it under aerobic conditions, being part of an O2 protection system (Kim and Burgess, 1996). NifZ is important for P-cluster maturation (Hu *et al*., 2007), and the function of NifT remains unknown (Thiel and Pratte, 2014). It has been suggested that electron transfer from the Fe protein to the MoFe protein within the protein-protein complex normally alterations conformational changes which enhance the affinity of the Fe protein for the MoFe protein (Lanzilotta *et al*., 1997). Nitrogenase activity was significantly influenced by different physiological variables, and when incubated under light, the rate of H_2 evolved by the fronds under anaerobic situations was remarkably higher than that under aerobic conditions, and H_2 uptake was significantly suppressed by both acetylene and carbon monoxide incubations (Kumar *et al*., 1990).

Action mechanism of nitrogenase

Nitrogenase is consists of two soluble proteins: component I and II. Component I known as MoFe protein or nitrogenase contains 2 mo atoms, 28 to 34 Fe atoms, and 26 to 28 acid-labile sulfides, also recognized as an iron-molybdenum cofactor (FeMoco). Component I is consisting of two copies each of two subunits (α and β); each subunit's stability based on the other in vivo. Component II known as Fe protein or nitrogenase reductase is including two copies of a single subunit. This protein has four non-heme Fe atoms and four acid-labile sulfides (4Fe-4S). Substrate binding and reduction takes place on component I, which binds to ATP and ferredoxin or flavodoxin proteins (Fdx or Fld) (Rees and Howard, 2000). Biosynthesis of nitrogenase FeMoco is an extraordinarily complex process that needs, minimally, the participation of nifS, nifU, nifB, nifE, nifn, nifV, nifH, nifD, and nifK gene products (Hu and Ribbe, 2011). Nitrogen fixation positively associated with carbon catabolism and biosynthesis, and nitrogen fixation negatively associated with photosynthesis at the proteomic level (Liu *et al*., 2021). NifB catalyzes the pivotal step of radical SAM-dependent carbide insertion

that happens concomitant with the insertion a "9th" sulfur and the rearrangement/coupling of two 4Fe-clusters into a complete 8Fe-core of the M-cluster (Hu and Ribbe, 2016). The hydrolysis of ATP contributes the energy for the reaction while the Fdx/Fld proteins supply the electrons. ATP is not hydrolyzed to ADP until component II transfers an electron component I. 21-25 ATPs are needed for each N_2 fixed. The association of nitrogenase component I and II and later dissociation happens several times to authorize the fixation of one N_2 molecule (Hallenbeck, 1992; Igarashi and Seefeldt, 2003; Tejera *et al*., 2004). Nitrogenase finally bonds each atom of nitrogen to three hydrogen atoms to form ammonia (NH_3). The nitrogenase reaction additionally produces molecular hydrogen as a side product, which is of notable interest for people trying to produce H_2 as an alternative energy origin to fossil fuels (Skizim *et al*., 2012; Yamamoto *et al*., 2014).

The nitrogenase enzyme complex (the nitrogen, fixing enzyme) is sensitive to O_2, that irreversible inactivates the enzyme, and diazotrophs must engage in mechanisms which on the other hand allow the supply of O_2 required for energy regeneration and protect Nase from the deleterious impacts of O_2, and they have developed diverse ways for limiting O_2 access to Nase: 1) It could avoid O_2 and live in environments which are constantly anaerobic, 2) Alternatively, it could generate a physical barrier around its Nase and in this way block O_2 from diffusing to the enzyme, 3) The microorganism could, but its metabolism, decrease the concentration of O_2 within the vicinity of Nasa, 4) They could adjust its Nasa in such manner as to render it resistant to inactivation by O_2 (conformational protection), 5) Finally, the microorganism could merely balance Nasa inactivation with the synthesis of new enzyme (Miller and Orme-Johnson, 1992; Soto-Urzua and Baca, 2001; Byer *et al*., 2015). Nitrogenase activity was always maintained even when population growth was influenced, and the stimulus may be very high during the stationary or death steps, conditions in which bacteria are frequently found in the environment (Richards, 1996; Barbosa *et al*., 2002). Nitrogenases are composed of two protein that can be purified individually: dinitrogenase and dinitrogenase reductase (Bulen and LeComte, 1966; Hageman and Burris, 1978; Barriere *et al*., 2001). Dinitrogenase, also related to as the MoFe protein or component I, is a 220- to 240-kDa tetramer of the nifD and nifK gene products that consists of two pairs of two complex metalloclusters known as the P-cluster and the iron molybdenum cofactor (FeMo-co) (Shah and Brill, 1973; Kim and Rees, 1992; Chan *et al*., 1993; Hu and Ribbe, 2013).

Each αβ pair of subunits of NifD and NifK includes one p-cluster and one molecule of FeMo-co (Rubio and Ludden, 2005). FeMo-co is consisted of homocitrate and a $MoFe_3$-S_3 cluster bridged to a Fe_4-S_3 cluster by three sulfur lignads (Rubio and Ludden, 2005). The Mo atom is correlated to the C-2 carboxyl and hydroxyl groups of R-homocitrate (Rubio and Ludden, 2005). In recent times, an electron-dense area has been found within the core of Fe atoms of FeMo-co and has been suggested to be a low-atomic-weight species (O or N) (Maeda *et al*., 1999; Einsle *et al*., 2002; Fay *et al*., 2007). The P-cluster is a [8Fe-7S] cluster with a structure akin to that of FeMo-co, which consists of two [4Fe-3S] cubanes associated by a central S atom (Austin and Lambert, 1994; Janas and Sobota, 2005; Rubio and Ludden, 2005). The P-cluster are located at the αβ subunit interface and are linked by cysteinyl residues from both subunits (Rubio and Ludden, 2005). The three variant nitrogenases differ in the composition of the active site and include either molybdenum, vanadium or only iron in the dinitrogenase component (Trncik *et al*., 2022). Each nitrogenase contains two core components, the catalytic dinitrogenses ($NifD_2K_2$, $VnfD_2K_2G_2$ or $AnfD_2K_2G_2$, respectively), and a dinitrogenase reductase, also known as the Fe protein (NifH, VnfH or AnfH) (Trncik *et al*., 2022). In all nitrogenase systems, Fe proteins couple the transfer of an electron gained from either a ferredoxin or a flavodoxin to their cognate dinitrogenase (MFe protein, with M = Mo, V or Fe) to the hydrolysis of two molecules of ATP per electron, and three-dimensional structure of the complex of the two constituents of Mo-nitrogenase, the Fe protein NifH and the MoFe protein NifDK; while, the complex is assembled transiently for each single electron transfer and the overall reaction demands at least 8 electrons per N_2 (Sellmann *et al*., 2000; Wilson *et al*., 2001; Corbett *et al*., 2004; Trnick *et al*., 2022). Nitrogenase is deeply inhibited by the environmental gas carbon monoxide (CO), and nitrogen-fixing bacteria depend on the protein CowN to grow in the presence of CO, and it has been

reported that CowN is a principal auxiliary protein in nitrogen fixation that engenders CO tolerance to nitrogenase (Medina *et al*., 2021).

nif gene products and their functions (proposed or known) in nitrogen fixation are nifH (Fe protein. Restrict electron donor to MoFe protein during nitrogenase turnover. NifH is also needed for FeMo-co biosynthesis and apo-MoFe protein maturation), nifD (α subunit of MoFe protein. Forms an $\alpha_2\beta_2$ tetramer with the β subunit. The site of substrate reduction, FeMo-co is within the α subunit of MoFe protein), nifK (β subunit of MoFe protein. P-clusters are present at each αβ subunit-interface), nifT (Unknown), nifY/nafY (Chaperone for the apo-MoFe protein. NafY is also a FeMo-co carrier and is suggested to aid in the insertion of FeMo-con into apo-MoFe protein), nifE (Forms $\alpha_2\beta_2$ tetramer with NifN. Essential for FeMo-co synthesis. Proposed to function as a scaffold on which FeMo-co is synthesized), nifN (Necessary for FeMo-co synthesis. Tetramer with NifE), nifX (Associated to FeMo-co synthesis. Accumulates an FeSMo-containing precursor), nifU (Molecular scaffold for the formation of Fe-S cluster for nitrogenase components), nifS (Active in mobilization of S for Fe-S cluster synthesis and repair), nifV (Homocitrate synthase, involved in FeMo-co synthesis), nifW (Active in stability of MoFe protein), nifZ (Unknown), nifM (Needed for the maturation of nifH), nifF (Flavodoxin. Physiological electron donor to NifH in *K. pneumoniae*), nifL (Negative regulatory element), nifA (Positive regulatory element), nifB (Needed for FeMo-co synthesis. Its metabolic product, NifB-co, is a specific Fe and S donor to FeMo-co), fdxN (Ferredoxin. In *R. capsulatus*, it serves as electron donor to nitrogenase), nifQ (Active in FeMo-co synthesis. Suggested for function in early MoO_4^{2-} processing), and nifJ (Pyruvate: flavodoxin (ferredoxin) oxidoreductase. Electron donor to Fe protein in *K. pneumoniae*) (Rubio and Ludden, 2005).

Rubio and Ludden (2005) reported that the Fe protein is important for the synthesis of functional P-clusters of the apo-MoFe protein and that the reaction controlled by the Fe protein promotes a conformational alteration within the apo-MoFe protein that leaves the FeMo-co insertion in site accessible. The apo-MoFe protein has been matured by the Fe protein is adapted by FeMo-co with no other demands, but it is labile unless stabilized by a third subunit that performs as a chaperone (NafY or NifY), and the maturation of the Fe protein needs the activity of NifM and the acquisition of a [4Fe-4S] cluster that would be directly donated by NifU. Soboh *et al*. (2010) reported that NifDK/NifB-co is an appropriate tool to obtain insights into the catalytic mechanism of nitrogenase; moreover, phylogenetic analysis of D and K homologs shows that several early emerging lineages, which consist of NifB, NifH, and NifDK encoding genes but which lack other genes needed for processing NifB-co into FeMo-co, might encode an enzyme with similar catalytic properties to NifDK/NifB-co. It is suggested that the level of magnesium available for complexation by the potent inhibitor ADP is the rate controlling different for nitrogenase activity (Dais and Kotake, 1980). In *Escherichia coli* the co-introduction of nifH and nifM lead to significant increase in levels of the nifH product, it proves that the nifH gene product alone is adequate for the assembly of an Fe protein-like structure in foreign prokaryotic and eukaryotic hosts (Berman et al., 1985). vnf gene products and their roles (proposed or known) in nitrogen fixation are vnf A (positive regulatory element), vnfE (proposed to function in FeV-co biosynthesis; analogous to NifE), vnfN (proposed to function in FeV-co biosynthesis; analogous to NifN), vnfX (Involved in FeV-co biosynthesis, accumulates an FeSV precursor to FeV-co), vnfH (vnf-Fe protein, obligate electron donor to VFe protein, also involved in FeV-co biosynthesis), vnfFd (Ferredoxin-like protein, putative electron donor to VnfH), vnfD (α subunit of VFe protein), vnfG (δ subunit of mature VFe protein. Possibly involved in the insertion of FeV-co into apo-VFe protein), vnfK (β subunit of VFe protein), and vnfY (Involved in FeY-co biosynthesis or insertion) (Rubio and Ludden, 2005).

Oxygen sensitive mechanism of nitrogenase

Oxygen can be either detrimental or beneficial for diazotrophs in organisms suitable for an aerobic catabolism, and it supports the production of a substrate for nitrogenase (ATP), but it can also impede the

activity and suppress the synthesis of this enzyme (Hill, 1988). Phylogenetic data shows that biological nitrogen fixation appeared in an anaerobic, thermophilic ancestor of hydrogenotrophic methanogens and later varied through lateral gene transfer into anaerobic bacteria, and ultimately aerobic bacteria including Cyanobacteria (Mus *et al*., 2019). Isotopic evidence proposed that nitrogenase activity remained at 3.2 Ga, prior to the appearance of oxygenic photosynthesis and rise of oxygen in the atmosphere, showing the presence of positive environmental conditions for oxygen-sensitive nitrogenase to evolve (Mus *et al*., 2019). Medina *et al*. (2021) reported that CowN's protection mechanism consists of decreasing the binding affinity of carbon monoxide (CO) to nitrogenase's active site nearly tenfold without interrupting substrate turnover, and CowN is a principal auxiliary protein in nitrogen fixation that engenders CO tolerance to nitrogenase. CO shows self-inhibition at partial pressures above 0.05 atm in Fe-nitrogenase, and CO shows significant inhibition of proton reduction in V- and Fe- nitrogenase (Harris *et al*., 2020). It has been suggested that oxygen inhibition of nitrogenase limited proterozoic global primary production, and oxygen levels enhanced when upright terrestrial plants separated nitrogen fixation in soil from photosynthetic oxygen production in leaves and shoots (Allen *et al*., 2019).

The Anf3 protein in the bacterium *Rhodobacter capsulatus* is important for diazotrophic growth with the iron-only nitrogenase, and Anf3 protects the iron-only nitrogenase from oxygen inactivation by operating as an oxidase in respiratory protection, with flavodoxin or ferredoxin as the physiological electron donors (Varghese *et al*., 2019). The coherent electron, proton, and N_2 transfer to the well-developed catalytic center will permit more effectual and versatile light-driven N_2-to-chemical conversions (Meng *et al*., 2021). Fe protein is decreased by ferredoxin (or dithionite *in vitro*) and transfers electrons to MoFe protein, which is integrated with ATP hydrolysis, and electrons are eventually transferred to dinitrogen at the catalytic site of MoFe protein resulting in the formation of ammonia molecules, and Fe protein is exceedingly sensitive to oxygen with half-life of less than 1 min because the [4Fe-4S] cluster is irreversibly destructed by oxygen (Nomata *et al*., 2006). Structural investigation of nitrogenase showed that N_2 activating MoFe-protein concentrates all its iron-sulfur clusters in a single active center having a high electron capacity (Shilov, 1987). Khademian and Imlay (2021) reported that oxygen also precisely injures the low-potential metal centers and radical-based mechanisms that increase anaerobic metabolism; therefore, committed anaerobes have evolved customized strategies that defend these different enzymes from occasional oxygen exposure. Kinetics of oxygen uptake suggested the existence of complex respiratory pathways in the cyanobacterium, and nitrogenase activity supported by respiratory activity with high affinity for oxygen, and as dissolved oxygen dropped below 10 μM with the onset of the dark phase, the nitrogenase activity failed to appear and organism could not grow under diazotrophic conditions (Misra, 1999). Raising the steady-state level of dissolved oxygen during the dark phase led to the appearance of the nitrogenase activity, commonly during the dark phase of the light/dark cycles (Misra, 1999). Oxygen scavenging is an important parameter in H_2 production enhancement, escalation of process parameters keeps oxygen and H_2 concentrations increase, and application of antioxidants and O_2 reducing substrates effectively boosts H_2 production (Asad Javed *et al*., 2022). Exposure to air caused the instant and irremediable inactivation of nitrogenase activity in an oxygen-sensitive mutant, selected strain 22Y, and inactivation was concomitant with the destruction of the molybdenum-iron (MoFe) protein of the nitrogenase complex (Smith *et al*., 1988). Oxygen-stimulation of nitrogenase activity was less showed in aerobic sediments around the rim of prawn burrow openings where subsurface sediments had been replaced to the surface by bioturbation (Tibbles *et al*., 1994). Nitrogenase, accountable for N_2 fixation in legume nodules, can be denatured by O_2 and functions under microaerobic situations; however, O_2 is needed for ATP production related to nitrogenase activity; as a consequence, O_2 concentration within infected cells is greatly regulated by a mixture of nodule respiration, leghaemoglobin, and an O_2 diffusion barrier (Guasch *et al*., 2001). Banerjee *et al*. (1989) reported that boosting concentrations of O_2 suppressed nitrogenase activity but 5% O_2 proved stimulatory for H_2 evolution in light, and in the dark, there was a moderate stimulation in H_2 evolution even up to 20% O_2. Nitrogenase is promptly destroyed by oxygen, in vivo and in vitro, so nitrogen fixing organisms face the difficulty of protecting their

nitrogenase from inactivation by oxygen (Gallon, 1981). Oxygen regulation is crucial in nitrogen fixation in rhizobia, and fixation is achieved by an oxygen-intolerant nitrogenase enzyme but needs respiration to meet its high energy demands (Rutten and Poole, 2019). The final stage of symbiotic establishment is activated by the NifA protein, conducted by oxygen at both the transcriptional and protein level (Rutten and Poole, 2019). It has been concluded that the protection of nitrogenase from O_2 damage, O_2 consumption at the cell surface is less effectual than generally considered, and it is found that alternative parameters like the supply of ATP and decreasing equivalents are more important (Oelze, 2000). The nitrogenase enzyme complex (the nitrogen, fixing enzyme) is sensitive to O_2, that irreversible inactivates the enzyme, and diazotrophs must employ mechanisms which, on the other hand, permit the supply of O_2 required for energy operate and protect Nase from the deleterious impacts of O_2 (Soto-Urzua and Baca, 2001).

It has long been known that N_2 fixation in the heterocyst can adopt to environmental O_2 concentration (Murry *et al*., 1984; Kangatharalingam *et al*., 1992), and it is considered that this may need an alteration of the glycolipid layer of the heterocyst to become a more or less effective gas diffusion barrier. A higher dark nitrogenase activity needs a higher rate of respiration and consequently a higher flux of oxygen, because dark nitrogenase activity is directly linked to aerobic respiration and was therefore applied as a measure of the influx of oxygen into the heterocyst (Stal *et al*., 2017). Drought stress seems to cause a decline in the maximum O_2-sufficient rate of nodule respiration or nitrogenase activity, and the alterations in nodule permeability reported to happen in drought-stressed nodules may be a response to elevated O_2 concentrations in the infected cell that may occur as nodule respiration declines (Del Castillo *et al*., 1994). The O_2 tolerance of nitrogenase was increased by introduction of uptake hydrogenase genes, showing this to be a functional method to increase nitrogenase enzyme activity under micro-oxic conditions (Liu *et al*., 2018).

Conclusions

Biological nitrogen fixation is the conversion of atmospheric nitrogen into ammonia, and is activated by nitrogenase, containing the MoFe and Fe proteins, which have binding sites for the mobile carrier of electrons, ATP, and a FeS cofactor, which accept electrons. Three distinct kinds of nitrogenase have been proved to exist in diazotrophs: the Mo-nitrogenase, the V-nitrogenase, and the Fe-only nitrogense. The [MoFe] nitrogenase system includes a homodimeric reductase constituent (Fe protein), and a tetrameric catalytic component (MoFe protein), and upon association of the two ingredients, an electron is transferred from the Fe protein 's [4Fe-4S] cluster to the MoFe protein 's M-cluster through the intermediate MoFe protein 'P-cluster; the cofactors which promote this electron transfer include the cubane-type [Fe-S] cluster, P-cluster [Fe_8S_7], and M-cluster [homocitrate: $MoFe_7S_9C$]. Mo is bound to 3 sulfur ions and two the OH and carboxyl group of 3-hydroxy-3-carboxy, adipic acid in the crystal structure. The evolutionary process for the development of nitrogenase arises from the ever-expanding biosphere and the constant state production of decreased nitrogen from abiotic sources. The nitrogen cycle has experienced highly changes. At beginning of life and offered an accurate anoxic scenario, the comparatively sufficient ammonium was probably utilized in an assimilation/mineralization cycle by protocellular organisms. To replenish ammonium pool, a vast N_2 reservoir obtaining from the primitive protosolar nebula was chemically decreased to ammonium at hydrothermal vents by FeS/H_2S of abiotic origin. Simultaneously, N_2 was also oxidized by atmospheric lightning to nitric nitrogen, which was applied as ammonium source via nitrite respiration by Nrf and/or nitrite and NO detoxification by incomplete denitrification (ancient Nar, Nir, and Nor). In the future, with the further study of the mechanism of nitrogen-fixing enzyme, it may be possible to artificially promote the evolution of nitrogen-fixing, so as to make more efficient use of biological nitrogen-fixing process.

Authors' Contributions

W.S.: writing-original draft preparations; M.H.S.: writing-original draft preparation, and editing. Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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