Mini-review

Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria

Ilana Berman-Frank a,*,1, Pernilla Lundgren b, Paul Falkowski a,c

a Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Rd., New Brunswick, NJ 08901, USA
b Department of Botany, Stockholm University, SE-106 91 Stockholm, Sweden
c Department of Geological Sciences, Rutgers University, 71 Dudley Rd., New Brunswick, NJ 08901, USA

Received 29 October 2002; accepted 23 December 2002
First published online 7 January 2003

Abstract

The biological reduction of N₂ is catalyzed by nitrogenase, which is irreversibly inhibited by molecular oxygen. Cyanobacteria are the only diazotrophs (nitrogen-fixing organisms) that produce oxygen as a by-product of the photosynthetic process, and which must negotiate the inevitable presence of molecular oxygen with an essentially anaerobic enzyme. In this review, we present an analysis of the geochemical conditions under which nitrogenase evolved and examine how the evolutionary history of the enzyme complex corresponds to the physiological, morphological, and developmental strategies for reducing damage by molecular oxygen. Our review highlights biogeochemical constraints on diazotrophic cyanobacteria in the contemporary world.

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Keywords: Cyanobacteria; Nitrogen fixation; Photosynthesis; Nitrogenase; Trichodesmium

1. Introduction

Although N₂ is the most abundant gas in Earth’s atmosphere, it is extremely unreactive. Before it can be incorporated into biological molecules, N₂ must be chemically reduced to the equivalent of ammonia. The biological reduction of N₂ is catalyzed by a multimeric enzyme complex, nitrogenase. This enzyme is irreversibly inhibited by molecular oxygen and reactive oxygen species (see summary in [55]). Oxygen stress on diazotrophic (nitrogen-fixing) organisms triggers a wide range of protective responses aimed at deterring the inhibitory effects of oxygen on nitrogenase. The level of resistance to oxygen stress and the mechanisms involved vary among diazotrophs and influence niche selection. The evolutionary trajectory of adaptive mechanisms that protect nitrogenase from molecular oxygen and reactive oxygen species can be discerned in physioecological patterns in microbial morphology, biochemistry, physiology, and community structure along a gradient from anaerobic to fully aerobic environments. The cyanobacteria are the only diazotrophs that actually produce oxygen as a by-product of the photosynthetic process, and which must negotiate the inevitable presence of molecular oxygen with an essentially anaerobic enzyme. Several recent reviews have focused on oxygen protection mechanisms and have examined molecular, phylogenetic, physiological, morphological, and regulatory adaptations [1,4,8,26,30,31,73]. Here we briefly examine how, on geological time scales, changing environmental conditions selected specific adaptations for diazotrophy and oxygenic photosynthesis in cyanobacteria, and how the evolution of cyanobacteria influenced Earth’s geochemistry.

2. Setting the background

2.1. The structure of nitrogenase

Nitrogenase appears to be a highly conserved enzyme complex. It is found in a diverse group of prokaryotes from the Bacteria and Archea [81,82], but is not encoded in any eukaryotic genome. Phylogenetic analyses suggest all extant nitrogenases sequenced so far are derived from a single common ancestor and that the catalytic subunits of the enzyme
complex indicate the enzyme existed prior to oxygenation of Earth’s atmosphere [10]. In many diazotrophs, nitrogenase comprises about 10% of total cellular proteins and consists of two components, an iron protein (Fe-protein) and an iron-molybdenum protein (MoFe-protein). The Fe-protein is a homodimer, composed of a single Fe₄S₄ cluster bound between identical ~32–40 kDa subunits. The Fe₄S₄ cluster is redox-active, and is similar to those found in small molecular weight electron carrier proteins such as ferredoxins [33]. It is the only known redox-active agent capable of obtaining more than two oxidative states [58] and transfers electrons to the MoFe-protein [11]. The MoFe-protein is an α₂β₂ heterotetramer; the ensemble is approximately 250 kDa. Each unit contains two types of clusters, a P cluster and a FeMoco center (sometimes called the M (magnetic) center). The P cluster is an Fe₈S₇ center that functions as a conduit for electron transfer, accepting electrons from the Fe₄S₄ cluster of the iron protein (in conjunction with ATP hydrolysis) and donating them to the FeMoco center (an inorganic structure of Fe₇MoO₄S₆ and an organic component, homocitrate), the site of substrate reduction [33]. Whereas both the Fe₄S₄ and P clusters are inactivated by O₂, the Fe₄S₄ cluster is much more susceptible and irreversibly damaged in vitro [55]. This is reflected by the difference in typical half-lives of the Fe-protein and MoFe-protein about 45 s and 10 min, respectively [31]. Complicating the situation is the fact that O₂ not only affects the protein structure but also inhibits the synthesis of nitrogenase in many diazotrophs. The repression is both transient (lasting only a few hours) and permanent [31]. Moreover, the toxicity of oxygen involves other forms of reactive oxygen species (ROS) that have both direct and indirect effects on nitrogenase and other components of the N fixation and assimilation pathways.

Alternative nitrogenases have been found that are homologous to the described enzyme, yet have vanadium or iron substituting for molybdenum [20]. The catalytic efficiency of these alternative nitrogenases is lower than that of the MoFe-nitrogenase; the specific activity of the VFe-nitrogenase is about 1.5 times lower than the MoFe-nitrogenase at 30 °C [45]. In addition to variations in metal cofactors, the nitrogenase complex is non-specific and reduces triple and double bond molecules other than N₂. These include hydrogen azide, nitrous oxide, acetylene, and hydrogen cyanide [11]. The non-specificity of this enzyme and the alternative nitrogenases containing other metal co-factors implicate the role of varying environmental pressures on the evolutionary history of nitrogenase that could have selected for different functions of the ancestral enzyme.

2.2. The historical setting

The function(s) of the primitive nitrogenases and the selective pressures on its evolution are debated and depend on the scenarios defining the early atmosphere and oceans, specifically the oxidation state and the form and concentration of greenhouse gases [24,70,71]. Physical models of solar radiation [54] suggest that the early atmosphere would have been mildly reducing with CH₄ and CO₂ serving as major greenhouse gases [15,39,40,53]. Under such conditions, several scenarios are proposed. In the first, ammonia could have been abundant initially and the primitive forms of nitrogenase may have evolved as a respiratory enzyme (N₂ being an accessible electron sink for anaerobic heterotrophs under the reducing conditions) or a detoxase utilized in detoxifying cyanides and other prevalent molecules in the ancient oceans [24,56]. Any increase in ultraviolet radiation would have caused rapid dissociation of NH₃ in the atmosphere with little fallout of ammonia to the oceans [22,39,40]. With the loss of free ammonia and cyanides, nitrogenase would have evolved to become the prevalent biological mechanism for nitrogen acquisition prior to the oxygenation of the atmosphere and the advent of nitrification [22,24]. Another possible scenario is that rapid weathering of fragments from bolide impacts, combined with efficient sequestration of carbon in the mantle, resulted in very low atmospheric CO₂ concentrations. These would have limited the amount of NO₃ formation from N₂ and CO₂, causing a crisis in fixed N that would select for nitrogen-fixation in the early Archaean eon ∼3.5 Giga annum before present (Ga) [40].

The change from an anoxic to an oxygenic atmosphere posed major challenges to cyanobacteria (and in particular to diazotrophic cyanobacteria). Present evidence based on paleosols and the isotopic fractionation of sulfur places the oxygenation of the atmosphere, resulting from cyanobacterial photosynthesis, between 2.4 and 2.2 Ga [25,32,38,66] (Fig. 1). Oxygenation of the atmosphere resulted in a change in the partial pressure of oxygen (pO₂), from <4 × 10⁻⁶ to >0.03 atm [54,60] (Fig. 1). With the increase in atmospheric oxygen, large-scale glaciation is documented, possibly resulting from the electrophyllic attack on CH₄ by an increased abundance of hydroxyl radicals [39]. These dramatic shifts would have influenced the global sinks of reduced nitrogen, phosphate and metal co-factors required for organismal growth. Phosphate availability may have limited primary production due to high adsorption of phosphate by iron-rich minerals. Consequently, sea-water phosphate concentrations are thought to have been more than tenfold lower than the present values (0.15–0.6 µM versus the modern value of 2.3 µM) [7]. Ammonia would be oxidized to nitrite and nitrate, and nitrification pathways would gain importance [23] (Fig. 1). The oxidation of ammonium to nitrate in the upper ocean and the concomitant hypoxic deep ocean would have facilitated a denitrification of the ocean, comparable to what is observed at the oxic/anoxic interface in the contemporary Black Sea.

In addition to the above constraints, the availability of required trace elements such as Fe, Mo, and V would also influence the evolution of nitrogenase. Pyrite-derived prosthetic groups in enzymes are especially vulnerable to damage by oxygen and reactive oxygen species (ROS) [77]; these lead to irreversible oxidation of the Fe–S clusters of the Fe₄S₄ protein. As described above, the nitrogenase enzyme is an
Fe-rich protein and the availability of iron influences N$_2$ fixation in cyanobacteria from its direct effect on Fe-rich protein synthesis, to effects on photosynthesis, growth and global productivity [5,22,51]. In the anaerobic environments of the Archean oceans, Fe would have been found predominantly in its reduced form (FeII) rather than FeIII (Table 1). In the Archean and Paleoprotozoic eons, the availability of Mo as a cofactor for nitrogenase would have been up to 90% lower (Table 1) due to its sequestration in sediments as insoluble sulfide mineral complexes [3,21,77]. Limited availability of Mo may have been exacerbated in the mid-Proterozoic, where weathering, under a moderately oxidizing atmosphere, would have enhanced the delivery of sulfate (SO$_4^{2-}$) to the deep ocean. Combined with primary production in the surface waters, this would have resulted in extremely high H$_2$S concentrations and removal of Mo via increased precipitation and formation of active thiomolybdate (MoS$_2^{2-}$) [3]. Alternative nitrogenases (with Fe or V replacing Mo) most certainly are older forms of the enzyme complex. Although, presently, Mo-independent nitrogenases have been found only in heterocystous diazotrophs (which are phylogenetically the most recently diverging group, see discussion below) but not in non-heterocystous species [4].

### 3. Nitrogenase in a changed environment: Multiple adaptations for a single purpose

Recent phylogenetic analysis of the two pairs of nitrogen fixation genes nifDK (encoding for alpha and beta subunits...
of nitrogenase) and nifEN (encoding for the two subunits of the NifNE protein complex involved in biosynthesis of the FeMo-cofactor) indicate that the enzyme’s evolutionary history predates the divergence of the Archaea and bacteria [24,78]. Fani et al. [24] propose a pathway of evolution for the ancestors of the nifDK and nifEN operons via two in-tandem paralogous gene duplication events. The divergence of a bicistronic operon controlling two genes would be consistent with either a neutral or mildly reducing Archean atmosphere.

During the course of planetary evolution, cyanobacteria have co-evolved with the changing oxidation state of the ocean and atmosphere to accommodate the machinery of oxygenic photosynthesis and oxygen-sensitive N₂ fixation. Various cyanobacteria go through a more complex pattern that can be traced phylogenetically and ecologically.

Although the abundance of anaerobic environments declined with the oxygenation of the atmosphere, many cyanobacteria have co-evolved with the changing oxidation state of the ocean and atmosphere to accommodate the machinery of oxygenic photosynthesis and oxygen-sensitive N₂ fixation. Various cyanobacteria go through a more complex pattern that can be traced phylogenetically and ecologically.

**Fig. 2.** Morphological and behavioral adaptations enabling nitrogen fixation for different cyanobacteria. Gray shaded areas indicate the localization of nitrogenase and black solid lines in the graphs designate nitrogen fixation rates. Photosynthesis is symbolized by the double dashed-line. Efficiency refers to a comparison of values given in the literature for activity measured under aerobic conditions (except for group I) as nmol ethylene reduced chl a⁻¹ h⁻¹. For some cyanobacteria the ability of nitrogen fixation has been lost, or was never present (bottom). Others (e.g., *Plectonema*) are capable of nitrogen fixation only under microaerobic conditions (I). Other cyanobacteria fix nitrogen during the dark period of a light/dark cycle (II). For 2 groups a cell specialization occurs (III and IV). Group III includes only two genera: *Trichodesmium* and *Katagnyemene*. For these, nitrogen fixation occurs during the light period, and nitrogenase is sequestered into only a fraction of the cells, often occurring consecutively [6,41,42]. Group IV compartmentalizes all heterocystous cyanobacteria, where nitrogen fixation occurs mostly during the light period, and where nitrogenase under aerobic conditions is found only in the heterocysts [2,8]. Although, under microaerobic conditions, nitrogenase can also be present in the other cells [64,66].

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tous species of cyanobacteria, these species fix nitrogen during the day and their mechanism of protection from photosynthetically evolved oxygen has puzzled researchers for many years [13]. In *Trichodesmium*, nitrogenase is compartmentalized in a fraction of the cells (typically between 10 and 20%) that are often arranged consecutively along the trichome [43]. However, active photosynthetic components (such as PSI and PSII complexes, Rubisco, carboxysomes) are found in all cells, even those harboring nitrogenase [6,28,29,34]. Hence, spatial aggregation of nitrogenase into certain zones/cells is not sufficient to protect against oxygen evolution. Indeed, there is evidence that a significant fraction of nitrogenase may be inhibited by O2 at any moment in time. Protection against oxygen in *Trichodesmium* was found recently to be a complex interaction between spatial and temporal segregation of the photosynthetic, respiratory and nitrogen fixation processes [6,17,43].

In *Trichodesmium*, a circadian clock controls the transcription of nitrogenase and the expression of photosynthetic genes essential to the activation of photosystems I and II (PSII) [16]. These genes (*psaA, psbA*) undergo a temporal phase difference with *nifHDK* expression of ~6 h, with *nifHDK* expression followed by *psbA psaA* [17]. Parallel measurements of changes in the activity of PSI, oxygen production, carbon, and N2 fixation reveal a temporal separation between N2 fixation and photosynthesis during the photoperiod. The period of high N2 fixation is characterized by a decline in gross photosynthetic production as well as enhanced light-dependent oxygen consumption and scavenging of oxygen by PSI via the Mehler reaction [36,37] (Gerchman et al., in preparation), which results in a negative net production of oxygen [6]. Cells can turn photosynthetic activity (seen as changes in cellular variable fluorescence) on or off within 10–15 min, illustrating that the photosynthetic machinery is not physically damaged. In contrast to fully evolved heterocystous cyanobacteria, all cells in a trichome are photosynthetically competent but individual cells modulate oxygen production and consumption during the photoperiod. Moreover, the increased occurrence of inactive photosynthetic zones during the hours of high N2 fixation provides evidence of both a temporal and spatial segregation of the two processes [6].

A highly refined specialization is found in heterocystous cyanobacteria (Fig. 2). Here, nitrogenase is confined to a micro-anaerobic cell, the heterocyst, which differentiates completely and irreversibly 12–20 h after combined nitrogen sources are removed from the medium. These cells are characterized by a thick membrane that slows the diffusion of O2, high PSI activity, the absence of PSII, and loss of division capability. Heterocystous organisms cannot obtain reductant directly from non-cyclic photosynthetic electron flow and rely on the supply of fixed carbon from adjacent vegetative cells for reducing equivalents. Cyclic electron flow around PSI supplies ATP (see recent comprehensive reviews on the formation and regulation of the heterocysts [1,2,8]). It was long thought that (a) heterocysts were required for nitrogen fixation, and that (b) the trigger for the formation of these specialized cells was N-deficiency. However, under anaerobic conditions some heterocystous cyanobacteria, such as *Anabaena variabilis*, can synthesize a different Mo-dependent nitrogenase (*Nif2*) in the vegetative cells [61,68,69]. *Nif2* is expressed shortly after nitrogen depletion but prior to heterocyst formation, and can support the fixed N needs of the filaments independently of *Nif1* nitrogenase in the heterocysts [67,69]. *Nif2* is also found in vegetative cells of non-heterocystous species [61]. Interestingly, a gene that is part of the *nif2* cluster in *Anabaena variabilis* ATCC 29413, *fdxH2* (encoding the [2Fe–2S] ferredoxin known as the direct electron donor to nitrogenase in heterocysts), has more residues in common and shares its oxygen sensitivity with the single *FdxH* from the non-heterocystous, filamentous cyanobacterium *Plectonema boryanum* PCC 73110 [65].

### 4. Evolutionary history of adaptive strategies for nitrogen fixation

The diverse strategies to overcome the oxygen dilemma reflect the wide-ranging flexibility and niches occupied by cyanobacteria: From anaerobic sediments and pore-waters to pelagic waters saturated with oxygen. It is also plausible to assume that the evolution of different strategies has been brought on by the increased oxygen pressure. While some paleontological evidence suggested that non-heterocystous cyanobacteria were present at 3.5 Ga bp [64], the validity of these results is questioned [9]. The earliest evidence of cyanobacteria is based on fossil lipid biomarkers, [66] that date cyanobacteria to at least 2.85 Ga bp. Evidence for heterocystous forms is found in akinete fossils dated at 1.5 to 2 Ga bp from cherts in West Africa and Siberia and silicified carbonates from Australia [63]. This fossil reconstruction is reflected in phylogenetic trees using functional genes involved in nitrogen fixation, such as *nifH* [76,80]. We constructed an unrooted phylogenetic tree of cyanobacterial representatives from the different adaptive strategies (Fig. 3). This tree suggests that adaptations evolved from anaerobic diazotrophs (i.e., *Phormidium, Lyngbya lagerheimii, Pseudanabaena PCC7403*) to microaerobic diazotrophs such as the filamentous *Plectonema boryanum* in which all cells can fix nitrogen with strict temporal separation from photosynthetic oxygen evolution only under continuous illumination. A full temporal separation, where nitrogen is only fixed at night, developed in unicellular cyanobacterial diazotrophs (*Cyanothecaceae ATCC51142, Gloeotrichia, Synechocystis* sp. WH8501) and some non-heterocystous filamentous diazotrophs (*Symploca*, see Ref. above). Further diversity in adaptation strategies also exists within the unicellular diazotrophs. In the genus *Cyanothecaceae*, for example, the seven described strains could fix nitrogen either aerobically or anaerobically [75]. Recent molecular phylogenies, for 33 strains of unicellular N2 fixers using...
small subunit ribosomal RNA sequences, showed no correlation between the phylogenetic relationships and the type of $N_2$ fixation [74]. Moreover, the authors hypothesize that the results of the phylogeny are indicative of multiple gains and/or losses of $N_2$ fixation abilities among the unicellular cyanobacteria [74]. Thus, the loss of cyanobacterial nif genes suggests that different strategies existed early, where some organisms were able to adapt to an oxic world, while others were not (e.g., Oscillatoria).

Tracing the phylogeny of cyanobacteria using nifH is problematic. The reconstructed trees are very sensitive to small changes in sequence. For example, Trichodesmium’s unique biological solution of combining a semi-temporal separation of $N_2$ fixation and photosynthesis with spatial heterogeneity appears in several trees as an early branch, suggesting a very ancient past [76,80,82]. Yet, in other trees (Fig. 3) it shows a later divergence. Moreover, organisms with the same strategy (e.g., Lyngbya and Cyanothecae) do not always cluster together (Fig. 3). Whether this is due to parallel evolution or an artifact of the phylogenetic reconstruction remains to be elucidated.

It is noteworthy, however, that the most recent phylogenetic branching (both for nifH and RNA trees) is observed for filamentous species where complete segregation of $N_2$ fixation and photosynthesis was achieved with the evolution of heterocystous cyanobacteria [79]. Heterocystous cyanobacteria are predominantly terrestrial, fresh water and coastal species, inhabiting eutrophic or brackish environments (e.g., Baltic Sea), with a few epiphytic and symbiotic representatives in the marine environment [52]. Very few heterocystous free-living species are found in the pelagic oceans though recently a novel species was found, designated Anabaena gerdi [14]. Relative to oceanic environments, fresh-water and coastal systems undergo more frequent and more extreme environmental changes so that the selective pressure on organisms in those environments would be higher and evolutionary adaptations more rapid, leading to the dominance of heterocystous cyanobacteria in these systems. In the large, slowly undulating world of the oceans, the ancient pathway adapted by Trichodesmium has persisted throughout the present time. This persistence suggests the tempo of evolution in marine cyanobacteria is extremely slow. Moreover, the relative evolutionary stability of nitrogenase across species reflects the flexibility of the enzyme in appropriating a variety of trace-metals as prosthetic groups, and its response to the inhibitory effects of molecular oxygen.

5. Future challenges—prospects

The nitrogen cycle of Earth is one of the most critical yet poorly understood biogeochemical cycles. Current estimates of global $N_2$ fixation are $\sim 240$ Tg N y$^{-1}$ with a marine contribution of 100–190 Tg N y$^{-1}$. Of this, a single non-heterocystous genus, Trichodesmium sp. contributes approximately $\sim 100$ TgN y$^{-1}$ (Capone pers. comm.). Geochemical evidence suggests that, on a global scale, nitrogen fixation does not always keep pace with denitrification on time scales of centuries to millenia [23], yet it remains unclear what process(es) limits nitrogen fixation in the oceans. More importantly, given the potential for heterocystous cyanobacteria to outcompete organisms such as Trichodesmium, it is unclear why the apparent tempo of evolution of marine diazotrophic cyanobacteria is so slow. Diazotrophic cyanobacteria have effectively become the “gate keepers” of oceanic productivity, yet despite the rapid radiation of eukaryotic oxygenic photosynthetic organisms throughout the Phanerzoic eon, marine cyanobacteria seem like living fossils. While molecular analyses and functional genomics may shed some light on this apparent paradox, we suggest that ultimately the answer lies in an understanding of cyanobacterial physiological ecology, which must itself be viewed from a paleoecological perspective. We are only just beginning to understand how biogeochemical cycles and microbes co-evolved on the planet.

Acknowledgements

Many thanks to John Reinfelder for help with the MINEQL+ calculations of iron speciation in Table 1, to
References


