Nitrogen isotope fractionation by alternative nitrogenases and past ocean anoxia

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Biological nitrogen fixation constitutes the main input of fixed nitrogen to Earth's ecosystems, and its isotope effect is a key parameter in isotope-based interpretations of the N cycle. The nitrogen isotopic composition ($\delta^{15}N$) of newly fixed N is currently believed to be ∼–1‰, based on measurements of organic matter from diazotrophs using molybdenum (Mo)-nitrogenases. We show that the vanadium (V)- and iron (Fe)-only "alternative" nitrogenases produce fixed N with significantly lower $\delta^{15}N$ (–6 to –7‰). An important contribution of alternative nitrogenases to N_2 fixation provides a simple explanation for the anomalously low $\delta^{15}N$ (<–2‰) in sediments from the Cretaceous Oceanic Anoxic Events and the Archean Eon. A significant role for the alternative nitrogenases over Mo-nitrogenase is also consistent with evidence of Mo scarcity during these geologic periods, suggesting an additional dimension to the coupling between the global cycles of trace elements and nitrogen.

stable isotopes | trace metals | paleooceanography | biogeochemistry

Biological nitrogen fixation contributes the bulk of new nitrogen to Earth's ecosystems. The isotope fractionation of nitrogen fixation (i.e., its isotope effect, ε_{fix}) is thus a key parameter for isotope-based studies of the marine and terrestrial N cycles (1, 2). ε_{fix}, often framed in terms of reaction rate coefficients, is most transparently defined here as $\delta^{15}N_{N2(aq)} - \delta^{15}N_{biomass}$, where $\delta^{15}N$ is $[(^{15}N/^{14}N_{sample}/^{15}N/^{14}N_{air}) - 1] \times 1{,}000$ when expressed in per mil (‰, parts per thousand). Currently, this parameter is assumed to be invariant with environmental conditions and equal to the in vivo isotope effect of nitrogen fixation by the Mo-nitrogenase [ε_{fix}^{Mo} is ∼+2‰ (3–6)], which is thought to be the most abundant in nature. As a result, the $\delta^{15}N$ of newly fixed N is ∼–1‰ (i.e., ∼+2‰ lower than the $\delta^{15}N$ of dissolved N_2 substrate, +0.7‰, Fig. 1*A*).

In addition to Mo-nitrogenase (the most common form of the enzyme), diazotrophs can possess two other nitrogenase isozymes (7). These so-called "alternative" nitrogenases differ chiefly from Mo-nitrogenases in that V or Fe replaces Mo in the active site. They also contain an additional protein subunit and exhibit slower kinetics compared with the Mo-nitrogenase (8). Such differences could result in distinct isotope effects, a possibility supported by Rowell et al. (9), who reported small but significant variations in biomass $\delta^{15}N$ from growth of wild-type diazotrophs possessing all three isozymes in media containing Fe and amendments of Mo, V, or neither metal. However, the use of multiple nitrogenase isozymes in the wild type (10) precludes the direct association between biomass $\delta^{15}N$ and the isotope effect of a particular isozyme. Here we (*i*) measured directly the isotope effects for Mo-, V-, and Fe-only nitrogenases using diazotroph mutant strains that could express only a single nitrogenase isozyme, (*ii*) determined the impact of metal limitation on alternative nitrogenase use and N isotope fractionation in wild-type bacteria, and (*iii*) provide several examples of how these results on N isotope fractionation may change our understanding of the N cycle in the past.

Results and Discussion

Isotope Fractionation During Nitrogen Fixation by Mo-, V-, and Fe-only Nitrogenases. We measured the in vivo isotope effect associated with each type of nitrogenase in two phylogenetically and metabolically distinct diazotrophic bacteria, *Rhodopseudomonas palustris* and *Azotobacter vinelandii*. *R. palustris* is an alpha-proteobacterium. It fixes N_2 anaerobically and was grown under anaerobic and photoheterotrophic conditions. *A. vinelandii* is a gamma-proteobacterium. It fixes N_2 aerobically and was grown under aerobic chemoheterotrophic conditions. All three nitrogenase isozymes are functional in these diazotrophs, allowing us to maintain the same genetic background while probing the effect of nitrogenase type on $\delta^{15}N_{biomass}$ (and thus ε_{fix}). To determine the isotope effect of each nitrogenase isozyme in the two diazotrophs, we used mutant strains that can only express a single nitrogenase isozyme due to genetic deletions or disruptions of the other nitrogenases (*Materials and Methods*). Batch cultures of mutants expressing the V- or Fe-only nitrogenase exhibited much higher fractionations than the mutants expressing the Mo-nitrogenase. The $\delta^{15}N_{biomass}$ of mutants (dark blue columns, Fig. 1) indicates that ε_{fix} for nitrogenases in *R. palustris* and *A. vinelandii* are, respectively, 2.94 ± 0.44‰ and 1.74 ± 0.22‰ for the Mo isozyme (Fig. 1*A*), 6.84 ± 0.34‰ and 6.33 ± 0.26‰ for the V isozyme (Fig. 1*B*), and 7.99 ± 0.25‰ and 6.87 ± 0.52‰ for the Fe-only isozyme (Fig. 1*C*). Fractionation in diazotrophs expressing the same isozyme does not vary significantly with organism phylogeny or energy metabolism. The absence of additional metabolic effects is also shown by similar $\delta^{15}N$ for *R. palustris* harvested in exponential and stationary phases (Fig. 1 *A*–*C*).

These data provide definitive fractionation measurements for the alternative nitrogenase isozymes and establish that the alternative nitrogenases have a much higher ε_{fix} than canonical Mo enzymes. Previously published data by Rowell et al. (9) (light blue columns marked by AvWT and AnWT in Fig. 1 *A*–*C*) derived from wild-type diazotrophs possessing multiple nitrogenases suggested a markedly lower ε_{fix} for the alternative nitrogenases

Significance

Biological nitrogen fixation is the main route by which nitrogen enters the biosphere. This reaction is catalyzed by nitrogenase, a metalloenzyme that exists in forms containing molybdenum, vanadium, or iron only. The contribution of the "alternative" vanadium and iron-only nitrogenases to nitrogen fixation in the present and the past is unknown. Here we show that the nitrogen isotopic composition (^{15}N to ^{14}N ratio) of biomass generated from nitrogen fixation by alternative nitrogenases is significantly and characteristically lower than biomass produced by molybdenum nitrogenases. In light of these results, nitrogen isotope measurements in ancient sediments imply an important role for iron-only nitrogenases in nitrogen fixation within certain anoxic, molybdenum-limited ancient environments.

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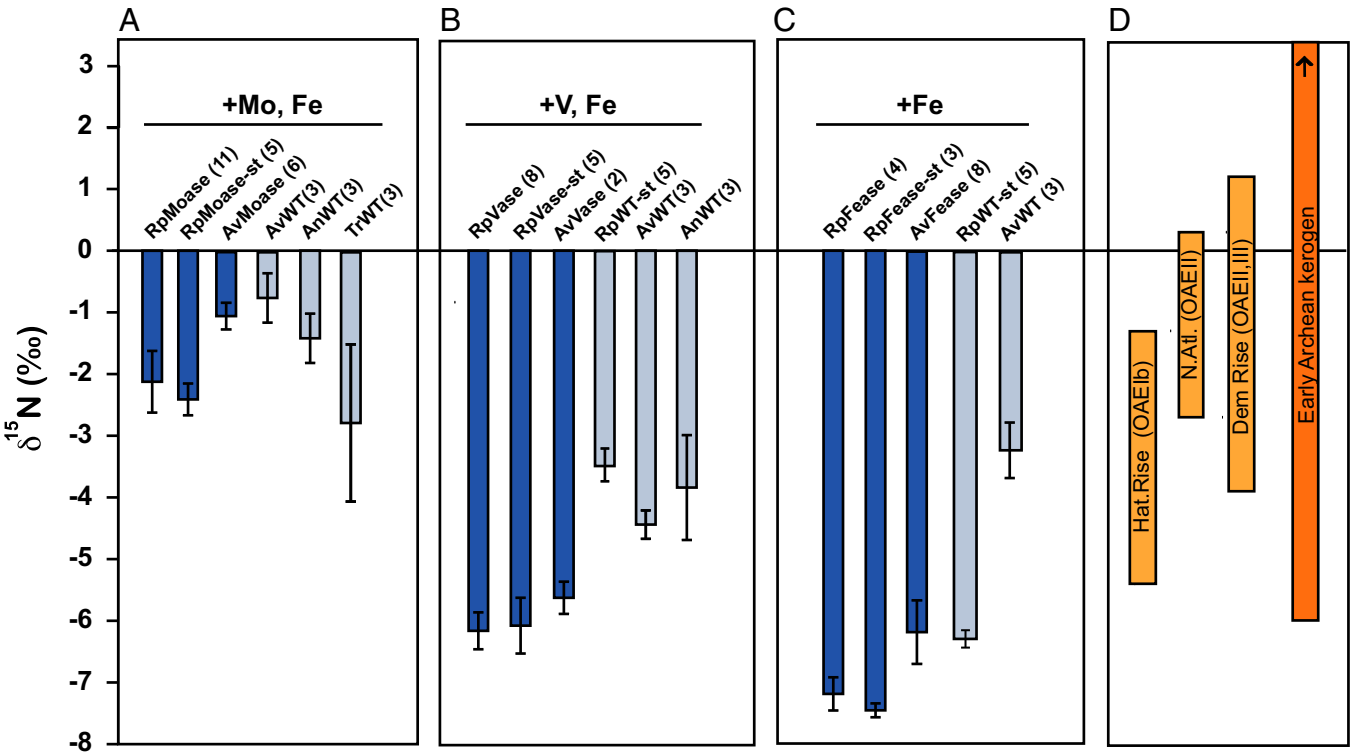

Fig. 1. δ^{15}N (in ‰ vs. air N_2) of biomass from diazotrophic growth of various bacteria (Rp, *Rhodopseudomonas palustris*; Av, *Azotobacter vinelandii*; An, *Anabaena variabilis*; Tr, *Trichodesmium*) using (*A*) canonical Mo-, alternative (*B*) V-, or (*C*) Fe-only nitrogenases in media amended with different metals (denoted by +Mo for 100 nM Mo, +V for 10 μM V, +Fe for 2.5 μM Fe). The δ^{15}N of mutant strains that express a single nitrogenase isozyme (Moase, Mo-nitrogenase only strain; Vase, V-nitrogenase strain; Fease, Fe-only nitrogenase strain) are indicated by dark blue bars; δ^{15}N of wild-type (WT) diazotrophs are indicated by light blue bars. Av, An, and Tr WT data were compiled from the literature (5, 9). The δ^{15}N of bacteria harvested in stationary phase is indicated by "st." Numbers in parentheses indicate culture replicates. Error bars are 1 SD. (*D*) displays the ranges in δ^{15}N measured in various Mesozoic OAE sediments (44, 45) (light orange bars) and in Early Archean kerogens (50) (dark orange bar). The upper value (indicated by arrow) for Archean samples (+13‰) is not shown. Data used in figure construction are in Tables S1 and S2.

[e.g., ~+4‰ for the Fe-only nitrogenase in *A. vinelandii* (Fig. 1)] compared to our measurements. This likely reflects the incomplete switch to alternative nitrogenases under metal limitation, as described below.

Given the lower in vitro specific activities of the V- and Fe-only nitrogenases compared with the Mo-nitrogenases (8, 11) and differences in their active sites, it is possible that the efficiency of N_2 reduction after the binding of N_2 to the active site is decreased, resulting in a greater proportion of N_2 unbinding (decreased commitment to catalysis) and thus more complete expression of the isotopic discrimination associated with the subsequent bond-breaking step in the process. It is also possible that a decrease in commitment to catalysis results from competition at the active between N_2 and H_2 (12, 13), as the latter is produced more abundantly by alternative nitrogenases (8). Additional studies on the intrinsic isotope effects of the nitrogenases and their expression at the levels of the enzyme and organism may help improve our understanding of the mechanism of N_2 reduction.

Metal Availability and Alternative Nitrogenase Use. The contribution of alternative nitrogenases to nitrogen fixation on local and global scales is currently unknown. Alternative nitrogenases are generally believed to be expressed only when Mo availability is low, possibly due to their lower specific activities and higher energy requirements (8). However, diazotrophic growth rates of *R. palustris* and *A. vinelandii* using V- or Fe-only nitrogenases in batch culture are ≥60% (and often >75%) of the rates associated with Mo-nitrogenase–based growth [*R. palustris*, Fig. S1; *A. vinelandii* (10)], implying that significant rates of N_2 fixation can be maintained by alternative nitrogenases. In terrestrial systems, asymbiotic nitrogen fixation is intermittently Mo-limited (14, 15). There is no evidence for Mo limitation in the modern open ocean. However, significant Mo scarcity was likely for certain marine environments in the geologic past (16, 17).

Ocean geochemistry during the "oceanic anoxic events" (OAE) of the Cretaceous Period (145–66 Mya) and the Archean Eon (4–2.5 Gya) (18, 19) would have provided conditions favorable for N_2 fixation by alternative nitrogenases. In the sulfide-rich OAE oceans (20), bioavailable molybdate would have been scavenged into sedimentary sulfides (21–23) and organic matter (24) via formation of particle reactive thiomolybdates (21–23) and reduced Mo-polysulfide species (25, 26). This would have lowered the concentration of Mo in seawater significantly (17, 27–31). Reinhard et al. have estimated ~10 nM Mo for ancient oceans containing significant zones of euxinia (17). This value is broadly consistent with measurements of <5 nM Mo beneath the chemocline in the Black Sea, a modern euxinic water body (32). Vanadium concentrations would have been lowered as well, although not as much as Mo [they are ~10 nM in Black Sea bottom waters (32)]. In contrast, anoxia generally tends to increase Fe bioavailability, as Fe(II)-sulfides are more soluble than Fe(III)-oxides (18). Thus, euxinic conditions would be favorable to Fe-only (and possibly V-) nitrogenases as important N_2 fixing enzymes (16).

Due to the near-absence of atmospheric oxygen in the Archean Eon, the mobilization of Mo and V by chemical weathering would have been slow, rendering the Archean ocean poor in Mo and V (17, 27). In contrast, the high solubility of Fe(II) in reducing environments would have resulted in an Archean

ocean rich in Fe (18). This condition, evidenced by the relative abundance of iron-rich Archean metasediments (e.g., banded iron formations) (18, 33–35), would have been particularly enhanced when sulfide production by bacterial sulfate reduction was limited by low levels of sulfate (36). High concentrations of dissolved Fe in the Archean ocean would have favored an Fe-based nitrogenase over a Mo-based nitrogenase. Whereas the evolutionary history of nitrogenase is a matter of ongoing debate (37, 38), it is possible that an Archean Fe-nitrogenase could be one form of an ancestral, cambialistic, "proto" nitrogenase that could bind one of multiple metals within its active site (39). This interpretation is consistent with the proposed early Proterozoic evolution of metal specific nitrogenases (40).

An increased contribution of alternative nitrogenases to N_2 fixation under Mo limitation should be reflected as a decrease in the $\delta^{15}N$ of fixed nitrogen. To determine directly the impact of low Mo availability on alternative nitrogenase use and the $\delta^{15}N$ of diazotroph biomass, we measured the $\delta^{15}N_{biomass}$ of wild-type *R. palustris* grown in low Mo medium (Fig. 2). Interestingly, *R. palustris* could be a representative diazotroph for OAEs: it fixes N_2 under anoxygenic phototrophic conditions and has millimolar sulfide tolerance. It also produces 2-methylhopanoid biomarkers (41), which have been found in OAE sediments (42). $\delta^{15}N_{biomass}$ of *R. palustris* decreased over time (Fig. 2). This cannot be explained by a variable ε_{fix} for the Mo-nitrogenase due to Mo limitation (43). Instead, this decrease reflects the simultaneous use of nitrogenase isozymes under metal limitation, as has been observed previously in *A. vinelandii* (10). With growth, the fraction of N_2 fixed by the Mo-nitrogenase, which was initially present due to intracellular Mo carried over in the cell inoculum as well as background Mo in the medium (3.7 ± 0.4 nM), decreased. Accordingly, the organism made increasing use of its Fe-only nitrogenase. Given measurements of barely detectable vanadium in the medium (1.8 ± 1.0 nM), significant use of the V-nitrogenase under Mo deficiency is unlikely. A decrease in $\delta^{15}N_{biomass}$ was also observed for wild-type cultures grown in Mo-limited, V-amended medium (Fig. S2). An increasing reliance on an alternative nitrogenase (Fe-only for Fig. 2, V for Fig. S2) is supported by the good fit between experimental data and a calculated prediction for $\delta^{15}N_{biomass}$ (black line, Fig. 2 and Fig. S2) based on dilution of the total Mo pool during cell growth (gray line, Fig. 2 and Fig. S2). Previous $\delta^{15}N_{biomass}$ measurements (Fig. 1 *B* and *C*, light blue columns for AvWT and AnWT cultures) for wild-type organisms *A. vinelandii* and *Anabaena variabilis* (9) also support our interpretation, as $\delta^{15}N_{biomass}$ in media with no purposeful amendment of Mo but also no steps to avoid background Mo are not as low as observed in mutants, suggesting only a partial shift to the alternative nitrogenases.

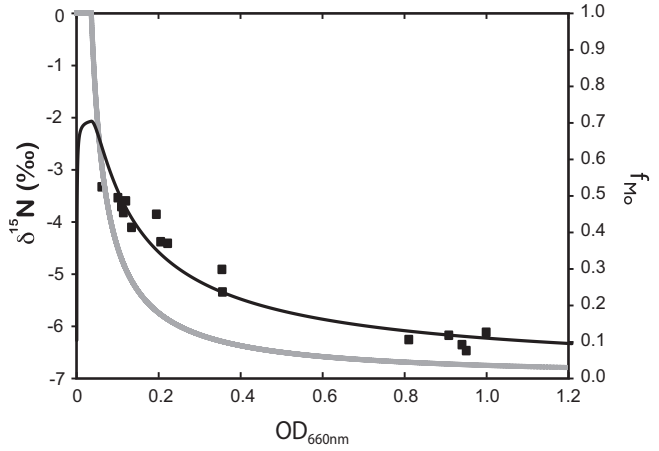

Fig. 2. $\delta^{15}N$ (in ‰ vs. air N_2) of biomass (measured, squares; modeled, black line) during batch growth of wild-type *R. palustris* in Mo-limited, N_2 fixing media ([Fe] = 2.5 μM, measured background [Mo] = 3.7 ± 0.4 nM, [V] = 1.8 ± 1.0 nM). Measurements are compiled from five replicate cultures. $\delta^{15}N$ was modeled over growth based on an isotopic mixing model that incorporates $X(t)$ (cell density over time), f_{metal} [gray line, fractional contribution of an isozyme to cell growth, expressed as $\gamma/X(t)$, where γ is a fitted parameter], and ε_{metal} (the isotope effect for a particular nitrogenase isozyme): $\delta^{15}N(t) = X(t-1)/X(t)\ \delta^{15}N(t-1) + [X(t) - X(t-1)]/X(t)]\ (\delta^{15}N_{N2} - f_{Mo}\ \varepsilon_{Mo} - f_V\ \varepsilon_V - f_{Fe}\ \varepsilon_{Fe})$. Values of ε_{Mo}, ε_V, and ε_{Fe} used were 2‰, 6‰, and 7‰, respectively. The model was initialized with $\delta^{15}N$ of –6.27‰ based on the assumption that for serial transfers in the same medium formulation, the $\delta^{15}N$ of cell inoculum is equivalent to the $\delta^{15}N$ of cells at the end of growth. The optimal γ-value is 0.036 (mean residual –0.0039 ± 0.33), assuming that Mo- and Fe-only isozymes are active.

Low $\delta^{15}N$ in Ancient Sediments and Its Significance for Alternative Nitrogenases. Certain organic-rich marine sediments from mid-Cretaceous OAEs (42, 44–47) and the early Archean (48–51), thought to be deposited under low Mo conditions (27, 31), have extremely low $\delta^{15}N$ (<–2‰, Fig. 1*D*). Values of $\delta^{15}N$ for organic-rich OAE sediments frequently fall below –1‰ and are always below +2‰ (42, 44–47). The $\delta^{15}N$ of kerogen-rich Early (Eo- and Paleo-) Archean cherts are more widely distributed (–7‰ to +13‰) (48–51). The bulk $\delta^{15}N$ of <~–2‰ observed in these geologic periods has not been observed in modern marine sediments (refs. 52–54 and references therein) and have been difficult to explain given the current model of the marine N isotope budget, which only includes N_2 fixation by the Mo-nitrogenase (2). As described below, the anomalously low $\delta^{15}N$ values can be explained by a decrease in the $\delta^{15}N$ of newly fixed N due to an important role for alternative nitrogenases, arising from the high-Fe, low-Mo conditions in seawater at those times.

The fixed nitrogen reservoir in the modern ocean is primarily subsurface nitrate, with a $\delta^{15}N$ of ~+5‰ (2). The $\delta^{15}N$ of the fixed N reservoir is determined by the fluxes of fixed N entering and exiting the ocean, which in today's ocean are dominated by N_2 fixation and denitrification [canonical denitrification and anaerobic ammonium oxidation, i.e., anammox (1)], the organism-level isotope effects of these processes, and the expression of these isotope effects at the environmental scale (2, 55). Based on measurements of the organism-level isotopic fractionation due to N_2 fixation by Mo-nitrogenase [ε_{fix}^{Mo} ~ +2‰ (3–6)], the $\delta^{15}N$ of newly fixed N has been assumed to be ~–1‰ (2). Canonical denitrification occurring in the sediments has little isotopic impact (ε_{SedDN} ~ 0‰) because nitrate is nearly completely consumed in the porewaters in which it occurs (2), although greater net fractionation does occur in some systems (56, 57). In contrast, the expressed fractionation associated with water column denitrification is substantial (ε_{WCDN} ~ +12‰ to +25 ‰), leaving fixed N enriched in ^{15}N (58), although it may be somewhat reduced from this at the global ocean scale (55). The isotope effect of anammox is +24‰ to +29‰ (59) and thus, like classical denitrification, leads to ^{15}N enrichment of the fixed N reservoir (*SI Text*, *Part I*). If there is no environmental-scale expression of the isotope fractionation associated with N loss pathways (as in the case of complete nitrate consumption by denitrification in the regions where it occurs), the $\delta^{15}N$ of the fixed N reservoir and any particulate sinking flux should decrease toward the $\delta^{15}N$ of newly fixed N by N_2 fixation (i.e., –1‰) (*SI Text*, *Part II*). Internal cycling processes such as nitrogen assimilation and remineralization (including nitrification) can lead to isotopically distinct fixed nitrogen pools (60–63). However, this cycling cannot directly impact the $\delta^{15}N$ of the whole ocean N reservoir, nor does it affect the annually integrated $\delta^{15}N$ of N exported from surface waters (ref. 61 and *SI Text*, *Part III*). In the modern ocean, water column denitrification raises the $\delta^{15}N$

of oceanic N well above that of N_2 fixation [by 6‰ to a $\delta^{15}N$ of ~+5‰ relative to atmospheric N_2 (2)].

A decrease in $\delta^{15}N$ during OAEs is consistent with an increased role of the Fe-only nitrogenase in the N cycle. Nearly complete nitrate consumption by denitrification associated with extreme anoxia [and euxinia (20)] would have resulted in little net isotope fractionation to raise $\delta^{15}N$. As fixed N loss processes went to completion, the $\delta^{15}N$ of the fixed N reservoir and of sediments would have thus converged on the $\delta^{15}N$ of N_2 fixation. In this isotopic framework, it is clear that N_2 fixation derived exclusively from Mo-nitrogenase (with an ε_{fix} of at most ~+3‰ based on measurements in *R. palustris*) cannot generate the $\delta^{15}N$ values <–2‰ measured for several OAE sediments (42, 44–47). In contrast, such values are easily explained by N_2 fixation with an alternative nitrogenase, which can have an ε_{fix} of up to ~+8‰ (Fig. 1*C*) and enable the ocean's fixed N reservoir to achieve a $\delta^{15}N$ as low as –7‰. For example, the extremely low $\delta^{15}N$ of –5.6‰ measured for an OAE1b sediment (45) implies that alternative nitrogenases could have been responsible for the majority of fixed N in this environment (>~70%). This may have reflected severe and prolonged Mo limitation of diazotrophy (Fig. 2). The more common, less extremely negative $\delta^{15}N$ values (i.e., up to –2‰) (42, 44–47) may be explained by only a partial contribution of alternative nitrogenases to N_2 fixation (e.g., less severe Mo-limitation) or a significant reliance on alternative nitrogenases coupled with less complete fixed N consumption by denitrification. The degree to which diazotrophs rely on their alternative nitrogenases for fixed N will depend on the environmental Mo concentration, the Mo concentration at which diazotrophs become Mo-limited, and the duration of metal limitation. Slight changes in Mo around its limiting concentration in euxinic oceans were likely (17), suggesting that diazotrophs only experienced periods of metal limitation intermittently. This metal physiology could have changed the relative importance of Mo- and alternative nitrogenases to N_2 fixation, potentially explaining some of the variability in OAE sediment $\delta^{15}N$ between –6‰ and –2‰.

Based on the geochemical arguments regarding environmental redox and trace metal speciation outlined above, we suggest that the Fe-only nitrogenase was a significant source of fixed N in the Cretaceous OAEs. Fe-only nitrogenase may have been used by cyanobacteria benefiting from the higher euphotic zone iron levels hypothesized to result from increased hydrothermal activity (e.g., refs. 64, 65). We note that the use of Fe instead of Mo in the FeFe cofactor of the Fe-only nitrogenase would result in only a small increase in the Fe requirement for this isozyme compared with Mo-nitrogenase (8, 66). Other possible diazotrophs using Fe-only nitrogenase include anoxygenic phototrophs living in a euxinic photic zone, a habitat predicted to occur within certain areas of the OAE ocean (67), and chemotrophs fixing N_2 within the aphotic, suboxic zone, as has been found in the Black Sea (68). Notably, iron fertilization of primary productivity has been proposed for OAEs (64, 65). The high rates of primary production previously proposed for the OAE could have been maintained by organisms using Fe-only nitrogenases because growth rates based on Fe-nitrogenases can be only moderately slower compared with Mo-nitrogenases [20% difference for *R. palustris*, Fig. S1; 40% difference for *A. vinelandii* (10)].

Higgins et al. (47) have offered an alternative explanation for decreased $\delta^{15}N$ commonly observed in OAE sediments that involves preferential shunting of low $\delta^{15}N$ ammonium to euphotic zone phytoplankton, with higher $\delta^{15}N$ ammonium being converted to nitrite, which is then denitrified completely. This scenario requires a greater isotope effect for ammonium assimilation than for ammonium oxidation. There is no modern analog to support this hypothesis, rendering it difficult to test. Studies of the isotope effect of ammonium assimilation suggest that it drops to ~5‰ as ammonia concentrations decrease to micromolar levels or below (69), as is characteristic of the modern upper ocean. All indications are that the isotope effect for ammonium assimilation will be ~10‰ lower than that for ammonium oxidation at similar ammonia concentrations (70). Thus, it seems unlikely that the suggested branching reaction of Higgins et al. (47) would preferentially shuttle low $\delta^{15}N$ nitrogen to phytoplankton whose biomass is exported to sediments.

Given the uncertain influences of long-term diagenesis on $\delta^{15}N$ and the poorly constrained biogeochemistry of Archean N cycling, it is difficult to make definitive statements on the causes of extremely low $\delta^{15}N$ values (<–2‰, reaching as low as –7‰) that have been measured repeatedly in kerogen-rich, early Archean sediments (48–51). Nonetheless, previous studies have attributed low values to biological processes, including N_2 fixation (50, 51). Given that the $\delta^{15}N$ of Archean atmospheric N_2 was similar to its modern day value (i.e., 0‰) (71), N_2 fixation occurring exclusively via Mo-nitrogenase is not consistent with sedimentary $\delta^{15}N$ values <–2‰, suggesting a contribution of alternative nitrogenases to the fixed N reservoir of the early Archean, as in the Cretaceous OAEs. In the anoxic environment of the early Archean, the fixed N reservoir would have been dominated by ammonium (50, 72). The absence of oxygen in the atmosphere precluded the rapid formation of nitrate and nitrite, and thus any losses through denitrification or anammox, which today act to raise the $\delta^{15}N$ of fixed N, could not occur. However, there may have been other N loss processes that fractionated the N isotopes [e.g., anaerobic ammonium oxidation by iron (III) reduction, Feammox (73)] and thus elevated the $\delta^{15}N$ of the N reservoir to some degree. If such processes were relatively unimportant, the isotopic composition of ammonium at the time, and thus the $\delta^{15}N$ of organic matter in sediments, may have more directly reflected the $\delta^{15}N$ of N introduced by N_2 fixation. In any case, the lowest $\delta^{15}N$ (–6‰ to –7‰) measured during the early Archean (48–50) are, as during the Cretaceous OAE, most consistent with N_2 fixation by an alternative nitrogenase (Fig. 1 *B* and *C*). Alternatively, the proposed low specific activity of proto-nitrogenases (39), possibly manifested as lower commitments to catalysis, would likely have resulted in a large N isotope fractionation by the enzyme, as observed here for V- and Fe-only nitrogenases. More positive $\delta^{15}N$ measured in late Archean sediments (≥0‰) has been interpreted to reflect the onset of isotopically fractionating N loss processes [classical denitrification or anammox (72, 74)] or some degree of high-temperature metamorphism, known to cause isotopic alteration in ammonium-containing silicates (51, 75). If the Fe-based nitrogenases continued to dominate N_2 fixation at that time, the net fractionation associated with N loss may have been even greater than previously reconstructed.

Alternative explanations for low $\delta^{15}N$ in the Archean are isotope fractionation during ammonium assimilation (50) and biosynthesis from strongly ^{15}N-depleted mantle sources of inorganic N (N_2 or ammonium) by chemosynthetic organisms inhabiting hydrothermal vents (76). The isotope effect associated with partial ammonium assimilation at high ammonium concentrations can generate very low $\delta^{15}N$ organic matter [$\varepsilon_{NH4\ assim}$ ~20‰ (69)]. However, if organic matter burial were the dominant output of fixed N, the expression of a high $\varepsilon_{NH4\ assim}$ in organic matter production would have raised the $\delta^{15}N$ of the ocean ammonium pool, effectively yielding the same integrated $\delta^{15}N$ export as if there were no fractionation associated with the process. The role of mantle-derived ammonium is very uncertain as the single report of low $\delta^{15}N$ [as low as –12.8‰ to –8‰ (77)] presents these values as preliminary due to possible analytical artifacts. The general observation that organisms in chemosynthetic-based hydrothermal ecosystems tend to have lower $\delta^{15}N$ (78) may reflect the use of alternative nitrogenases in these sulfidic (and thus potentially Mo-poor) systems.

Conclusions

Our data show much stronger N isotope fractionation for N_2 fixation by alternative V- and Fe-only nitrogenases than by canonical Mo-nitrogenases. Importantly, our results decrease the lower bound of $\delta^{15}N$ that can be achieved for the ocean fixed N reservoir to ~–7‰. The simultaneous use of Mo and alternative nitrogenases or incomplete fixed N loss processes would tend to increase $\delta^{15}N$ in sediments above this potential minimum, allowing the reconstruction of more plausible N cycle scenarios to explain sedimentary $\delta^{15}N$ between –7‰ and –2‰. N_2 fixation by alternative nitrogenases hence provides an attractive explanation for the low $\delta^{15}N$ measured in OAE and Archean sediments that is consistent with the expected metal chemistry and cycling of those times. It has been suggested that Mo scarcity may have constrained Precambrian ocean productivity through N limitation, slowing the rise in atmospheric oxygen (16, 17). Our findings are indeed consistent with an effect of metal availability on N_2 fixation in the Precambrian ocean. However, we suggest that the use of alternative nitrogenases may have largely compensated for the proposed restrictions on N_2 fixation by Mo scarcity.

Although the existence of alternative nitrogenases has been known for the past 30 years (79), their potential role in environmental N cycling has been largely disregarded. However, the persistence of genes coding for alternative nitrogenases in the environment (80) suggests an ongoing role in N_2 fixation. Significant N_2 fixation by the alternative nitrogenases would require fundamental changes in the interpretation of ocean and terrestrial nitrogen isotope data. For example, a lower $\delta^{15}N$ for newly fixed N would imply that the net fractionation associated with total denitrification in the modern ocean is greater than previously estimated. A greater net fractionation on the whole-ocean scale would call for a downward revision in the ratio of sedimentary to water column denitrification and thus a lower rate for total oceanic denitrification (2). This reinterpretation would be in the correct direction to reduce the apparent current imbalance between N_2 fixation and denitrification (2). The question of the importance of alternative nitrogenases in oceanic and terrestrial ecosystems warrants further research and may lead to a new understanding of the global N cycle and its links to trace element cycling. The $\delta^{15}N$ of newly fixed N may help in this effort by identifying N_2 fixation by the alternative nitrogenases.

Materials and Methods

R. palustris strains CGA009 (wild type), CGA753 (Mo-nitrogenase only, V-nitrogenase ΔvnfH Fe-only nitrogenase ΔanfH), CGA766 (V-nitrogenase only, Mo-nitrogenase ΔnifH nifD::Tn5 Fe-only nitrogenase ΔanfA), and CGA755 (Fe-nitrogenase only, ΔnifH ΔvnfH) were grown in batch culture under anaerobic photoheterotrophic conditions in defined nitrogen fixing medium containing 10 mM succinate (81). Metals were added as Na_2MoO_4 (to a final concentration of 100 nM, measured as 95.4 ± 3.6 nM Mo) and $NaVO_3$ (to a final concentration of 10 μM, measured as 8.5 ± 0.3 μM V). Total Fe concentration in the media (2.5 μM, measured as 2.1 ± 0.3 μM) was not varied. Average background Mo and V levels in media with no Mo and V additions were 3.7 ± 0.4 nM and 1.8 ± 1.0 nM, respectively. Background Mo was 7.2 ± 3.7 nM in V-amended media. *A. vinelandii* strains OP (wild type), CA1.70 (Mo-nitrogenase only, V-nitrogenase ΔvnfDGK::spc Fe-only nitrogenase ΔanfHD70::kan), CA11.70 (V-nitrogenase only, Mo-nitrogenase ΔnifHDK ΔanfHD70::kan), RP1.11 (Fe-nitrogenase only, ΔnifHDK ΔvnfDGK::spc) were grown under aerobic batch culture conditions in defined nitrogen fixing medium (10) with 5 μM Fe amended with 100 nM Mo or 100 nM V. Metal concentrations (see above) and diazotrophy were verified periodically with inductively coupled plasma-mass spectrometry and acetylene reduction assays (82). Bacterial growth was monitored spectrophotometrically as optical density at 660 nm (*R. palustris*) and 620 nm (*A. vinelandii*). The $\delta^{15}N$ of bacterial biomass collected onto combusted glass fiber filters throughout growth was measured by gas chromatography-isotope ratio mass spectrometry at the Rutgers Stable Isotope Facility (New Brunswick, NJ). Isotopic data are tabulated in Tables S1–S3.

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