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# Mechanistic Insights on Cellular Nitrogen Isotope Fractionation by N2-reducing Mo-nitrogenase

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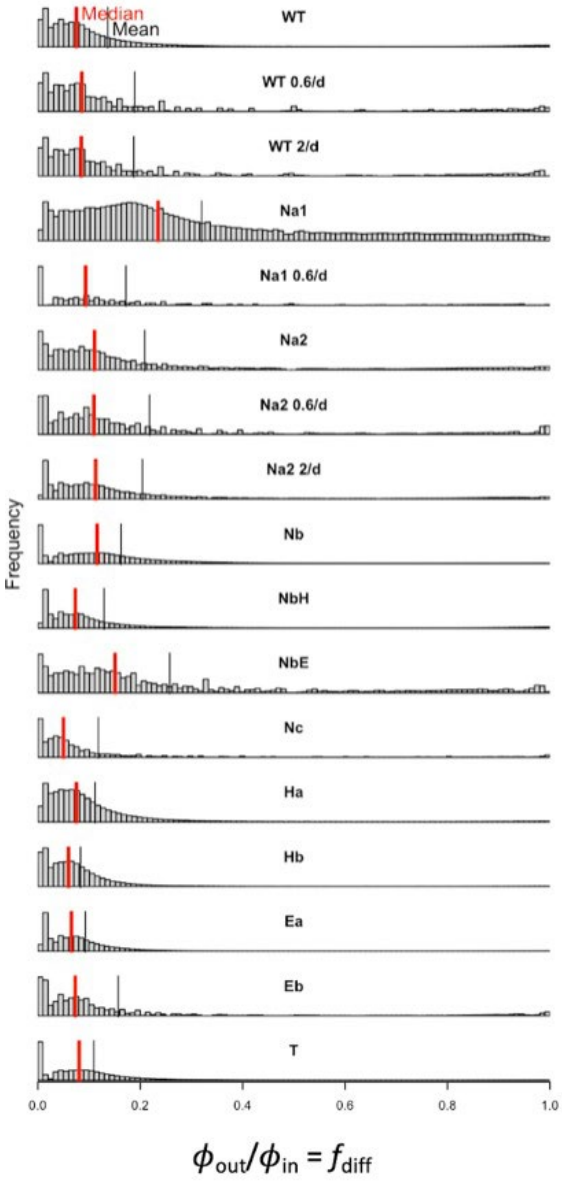
The nitrogen stable isotope (15N/14N) composition of biomass is a powerful tool for reconstructing N cycling, but its interpretation depends on understanding the 15N-fractionation of biological nitrogen fixation (BNF; $\varepsilon$ = δ15N_dissolved_N2 - δ15N_N2_fixer_biomass where δ15N reflects 15N/14N). It remains unknown specific causes why molybdenum nitrogenase BNF leads to low $\varepsilon$ values (~1-2 ‰) while BNF by less abundant alternative vanadium and iron-only isoforms result in larger $\varepsilon$ values (~5-7 ‰). Potential reasons include (1) variations in nitrogenase isoform KIE and (2) variations in cellular scale expression of the kinetic isotope effect (KIE) of nitrogenase due to changes in reaction reversibility. To better understand how nitrogenase structure-function constrains $\varepsilon$, we examine $\varepsilon$ sensitivity to variations in key functional residues for N2, proton, or electron delivery to the Mo-nitrogenase active site in the model nitrogen fixer *Azotobacter vinelandii* using batch and chemostat cultures of wild type and nitrogenase mutant strains. Values of $\varepsilon$ varied from 2 to 7 ‰, with largest $\varepsilon$ variations observed for N2 substrate channel mutants. Using an isotope fractionation model constrained by nitrogenase reaction mechanism, the data suggest that low $\varepsilon$ values reflect low reversibility of net N2 diffusion into the Mo-nitrogenase active site (_out/_in = *f*_diff < 20 %), leading to limited cell-scale expression of a large intrinsic KIE for N2 reduction ($\varepsilon$ >10‰). This interpretation is consistent with the lack of a global correlation between $\varepsilon$, growth rate, and specific activity across culture conditions and strains, suggesting that the rate limiting step of BNF shifts between isotopically sensitive and insensitive steps of the nitrogenase reaction mechanism. Highly conserved amino acid residues for the N2 channel of Mo-nitrogenase among all natural extant variants supports the preponderance of low $\varepsilon$ for Mo-nitrogenase BNF.




$$f_{diff} = \phi_{out}/\phi_{in} \qquad f_{cat1} = \phi_2/\phi_1$$

$$^{eq}\alpha_{diff} \qquad ^{eq}\alpha_{cat1}$$

$$\boldsymbol{N_2\ (cytosol)} \underset{\alpha_{out} = {}^{eq}\alpha_{diff} \cdot \alpha_{in}}{\overset{\alpha_{in}}{\rightleftarrows}} \boldsymbol{N_2\ (active\ site)} \underset{\alpha_2 = {}^{eq}\alpha_{cat1} \cdot \alpha_1}{\overset{\alpha_1}{\rightleftarrows}} \boldsymbol{N_2H_2\ (active\ site)}$$

$$\alpha_{obs} = \{\alpha_{diff}^{eq} \times [\,(\alpha_{cat1}^{eq} \times \alpha_3 - \alpha_1) \times f_{cat1} + \alpha_1] - \alpha_{in}\} \times f_{diff} + \alpha_{in}$$

$$\therefore f_{diff} = \frac{\alpha_{obs} - \alpha_{in}}{\alpha_{diff}^{eq} \times [\,(\alpha_{cat1}^{eq} \times \alpha_3 - \alpha_1) \times f_{cat1} + \alpha_1] - \alpha_{in}}$$