Nitrogenase loosens its belt to fix dinitrogen

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Title:
Nitrogenase loosens its belt to fix dinitrogen.

Standfirst:
Nitrogenase reduces dinitrogen at its iron-sulfur core to produce ammonia by a convoluted mechanism. Now, research highlights the importance of sulfur mobility on nitrogenase's metallocofactor for nitrogen fixation.

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Main body:
Elemental nitrogen is essential to all life on the planet, being quite literally part of our DNA. Some bacteria and archaea can produce reduced and useable forms of N by fixing kinetically inert atmospheric dinitrogen (N_2) to ammonia (NH_3).[1] This challenging reaction is exclusively achieved by way of the complex two-component metalloenzyme nitrogenase. For the Mo-dependent nitrogenase from *Azotobacter vinelandii* (V-dependent and all-Fe alternatives exist), an ATP-dependent reductase known as the Fe protein (also known as Av2) delivers electrons to the N_2-reducing MoFe protein (also known as Av1). Importantly, nitrogenase also produces molecular hydrogen (H_2) while fixing N_2, making it a biocatalyst of significant biotechnological interest.

It goes without saying that the precise mechanism of biological N_2 fixation has attracted much attention, with research efforts spanning several decades. The catalytic heart of the MoFe protein is the FeMo-cofactor (or FeMoco: [7Fe-9S-C-Mo-*R*-homocitrate]) on which N_2 fixation occurs. In 2020, Hu, Ribbe and coworkers reported the X-ray crystal structure of MoFe protein isolated from *A. vinelandii* cultured under N_2-fixing conditions, where the commonly used reductant dithionite was excluded such that nitrogenase turnover was arrested during purification.[2] This in-progress MoFe protein (MoFe*) appeared to enable the observation of a dinitrogen species present on the FeMoco for the first time (**Figure 1**). Importantly, belt sulfurs around the FeMoco were found to be displaced to accommodate this dinitrogen species, an action previously observed when associating other ligands (*i.e.*, carbon monoxide, selenocyanate) to the FeMoco and alternative V-dependent cofactor FeVco.[3] These findings were subsequently contested, leading to the reporting authors' conclusion that further studies were required to confirm the nature of MoFe*.[4,5] Now writing in *Nature Catalysis*, this team reports in-depth EPR spectroscopy as well as catalysis with isotopically labeled substrates to confirm that MoFe* does indeed proceed to form NH_3 in the absence of additional N_2, implying that the previously reported dinitrogen species may indeed be relevant to nitrogenase catalysis.[6] The researchers also observed that while this species can be chased off of FeMoco (to produce NH_3), importantly, a replacement sulfur source was required for continued activity. This highlights the importance of belt sulfur mobility and draws attention to a seemingly important yet previously unrevealed mechanistic step.

In the prominent Lowe-Thorneley kinetic model, N_2 fixation involves the activational reductive elimination of one H_2 equivalent from the FeMoco per N_2 fixed, explaining the persistent production of H_2 even with an N_2 partial pressure of >50 MPa.[1,7] In reverse, H_2 (or molecular deuterium, D_2) can

undergo oxidative addition to the FeMoco and displace FeMoco-associated N_2; subsequent protonolysis of the resulting metal-deuterides (M-D, introduced by D_2) in water yields HD, a reporter of having achieved sufficiently activated and N_2-associated FeMoco.[8] Since the isolated MoFe* protein was thought to contain N_2-associated FeMoco, the authors treated this protein with D_2 to displace N_2 and introduce M-Ds to FeMoco. Successive treatment with acetylene (C_2H_2), an alternative non-physiological substrate of nitrogenase, yielded partially deuterated ethylene (C_2H_3D) and is consistent with MoFe* having contained N_2-associated FeMoco.[9] The authors also employed EPR spectroscopy to characterize the MoFe* protein in comparison to resting state MoFe protein, as well as MoFe* protein subjected to turnover conditions to reduce N_2 and return to the resting state. In contrast to the resting state MoFe protein, MoFe* exhibited three new features in the $S = ½$ region at $g = 2.22$, 2.01 and 1.88, which were attributed to N_2-bound FeMoco. Importantly, these features were lost after turnover of MoFe* with the Fe protein/ATP/dithionite under Ar, once again consistent with MoFe* being a catalytically relevant intermediate state.

The researchers also sought to liberate the N_2 associated to FeMoco by resuming MoFe* catalysis to yield NH_3. MoFe* protein isolated from *A. vinelandii* cultured on $^{15}N_2$ was subjected to enzymatic turnover under Ar (the only source of $^{15}N_2$ being already associated to the FeMoco). The subsequent production of $^{15}NH_4^+$ was confirmed by frequency selective ^{1}H-NMR to originate only from $^{15}N_2$-loaded MoFe*, once again consistent with MoFe* being a catalytically relevant intermediate. Further, acid-quenching of $^{15}N_2$-charged MoFe* led to the evolution of $^{15}N_2$, as detected by GC-MS. Importantly, the authors noted a significant presence of $^{14}NH_4^+$ during these and control experiments, which was attributed to instability and degradation of the Fe protein. By this logic, it could also be proposed that the observed $^{15}NH_4^+$ originated from degradation of the MoFe* protein synthesized under $^{15}N_2$ diazotrophic conditions (*i.e.*, $^{15}N_2$ incorporation within the MoFe* protein's amino acids during diazotrophic growth), although control, non-turnover experiments of $^{15}N_2$-loaded MoFe* did not yield $^{15}NH_4^+$, and acid-quenching of $^{15}N_2$-loaded MoFe* produced $^{15}N_2$, which would not be liberated upon the degradation of ^{15}N-containing amino acids of $^{15}N_2$-loaded MoFe*.

Dithionite is commonplace in activity assays of nitrogenases although it is considered to be complex and potentially non-innocent, decomposing into compounds such as sulfide (S^{2-}), sulfate (SO_4^{2-}) and sulfite (SO_3^{2-}). Here, Hu, Ribbe and coworkers noted, remarkably, that dithionite-free MoFe* could not recover its activity when using the alternative sulfur-free reductant Eu(II)-EGTA. Interestingly, activity could be recovered in the presence of SO_3^{2-} or selenite (SeO_3^{2-}) (but not for S^{2-} or SO_4^{2-}), pointing to an additional (and perhaps non-physiological) role of dithionite in nitrogenase *in vitro* activity assays (**Figure 1**). Since oxidized sulfur precursor species have not yet been identified on the FeMoco, the authors have proposed that a previously unconsidered reductive pathway is necessary to charge the FeMoco with sulfur. Indeed, the total flux of electrons used by nitrogenase is observed to decrease when reducing both N_2 and H^+ *vs*. H^+-only reduction.[10] Replacing dithionite as the bulk electron donor enables the precise determination of the electrons consumed by nitrogenase, and while confirming that electron flux through nitrogenase is in fact uninhibited during N_2 fixation, sub-optimal values of electron flux towards N_2 fixation (55-59%) have been observed.[11,12] Interestingly, 23% of electrons delivered to the nitrogenase complex were previously unaccounted for, echoing the 21% of electrons that were not accounted for in the present study.[6,12]

In summary, Hu, Ribbe and coworkers provide evidence for the release of N_2/NH_3 by MoFe*, supporting their earlier observation of a catalytically relevant N-species bound to the FeMoco upon the exclusion of dithionite during MoFe protein purification.[2] In addition, they also show that dithionite-free MoFe protein requires a sulfur source (SO_3^{2-} shown here) to recover its activity, and propose mechanisms for the previously unconsidered reductive charging of FeMoco with sulfur for catalysis (**Figure 1**). Could the ~22% of "missing" electrons (perhaps two electrons for each N_2 fixed?) indeed be required to charge the FeMoco with sulfur? Could another physiologically relevant sulfur species act as the source of FeMoco sulfur? Future dithionite-free electron inventory studies will be required to identify the destination of these elusive electrons.

Competing interests

The author declares that there are no competing interests.

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FIGURE CAPTION

Figure 1. An additional role for the non-physiological reductant dithionite during *in vitro* nitrogenase catalysis. Representation of nitrogen fixation in one half of the $\alpha_2\beta_2$ MoFe heterotetramer (optimal stoichiometries) from *Azotobacter vinelandii*, adapted from PDB files 6UG0 (MoFe protein) and 1FP6 (Fe protein). In the mechanism proposed by Hu, Ribbe and coworkers, dithionite could serve as both the electron donor and sulfur source in *in vitro* assays.[6] Fe = rust, S = yellow, C = gray, N = blue, O = red, Mo = cyan, P = orange and Mg = green. PDBs were manipulated using ChimeraX and the figure was prepared using Affinity Designer.