Comment on “Structural evidence for a dynamic metallocofactor during N₂ reduction by Mo-nitrogenase”

John W. Peters¹*, Oliver Einsle²*, Dennis R. Dean³, Serena DeBeer⁴, Brian M. Hoffman⁵, Patrick L. Holland,⁶ Lance C. Seefeldt⁷

¹Institute of Biological Chemistry, Washington State University, Pullman, WA 99164, USA. ²Institute of Biochemistry, Albert-Ludwigs Universität, Freiburg, Germany. ³Biochemistry Department, Virginia Tech, Blacksburg, VA 24061, USA. ⁴Max Planck Institute for Chemical Energy Conversion, D-45470 Mülheim an der Ruhr, Germany. ⁵Department of Chemistry, Northwestern University, Evanston, IL 60208, USA. ⁶Department of Chemistry, Yale University, New Haven, CT 06520, USA. ⁷Chemistry and Biochemistry, Utah State University, Logan, UT 84322, USA.

*Corresponding author. Email: jw.peters@wsu.edu (J.W.P.); einsle@bio.chemie.uni-freiburg.de (O.E.)

Kang et al. (Reports, 19 June 2020, p. 1381) report a structure of the nitrogenase MoFe protein that is interpreted to indicate binding of N₂ or an N₂-derived species to the active-site FeMo cofactor. Independent refinement of the structure and consideration of biochemical evidence do not support this claim.

Understanding the mechanism of the complex metalloenzyme nitrogenase, the sole biological catalyst for N₂ reduction to NH₃, is of fundamental interest and has the potential for practical importance. This endeavor has challenged top scientists in the fields of bioinorganic chemistry and mechanistic enzymology for more than half a century. The structure reported by Kang et al. (1) and described as trapped under physiological N₂ turnover conditions, with the interpretation of an N₂-derived species trapped at the active site, would be an important advance. However, its nature and implications were surprising and troubling to us in several aspects. Nitrogenase presents several challenges experimentally, and such a feat would require overcoming barriers that many in the community are still challenged with in solution work. There have been many advances in understanding the mechanism of nitrogenase in the past decade, and we argue that a wealth of biochemical data strongly indicates that it should not be possible to generate a trapped state by treating the samples in the way described in the paper (2–4).

It is claimed that purifying the protein in the absence of artificial reductants results in the capture of an N₂ turnover state that contains the P-clusters in an oxidized state termed Pox. In the absence of external reductants, the MoFe protein oxidizes, with the P-cluster going to the Pox state. This was how the crystals were generated for the first structure of the Pox state in 1997 (5). The observation of the Pox state does not provide evidence for the involvement of this state in catalysis, as implied by the discussion in Kang et al., but rather points to an oxidized protein, with the FeMo cofactor also possibly oxidized. The electronic states of both P-clusters and FeMo cofactors can be determined by standard perpendicular-mode electron paramagnetic resonance spectrum. Without such data, it is not possible to distinguish the reported state from the oxidized MoFe protein.

Stimulated by these concerns, we reanalyzed the reported structure. Kang et al. propose that the crystallographic data support the binding of a N₂-derived species in place of belt sulfurs in the FeMo cofactor. N₂ and S² (the anticipated species of S in the FeMo cofactor) are nearly isoelectronic and thus require high-quality data to differentiate. Using the structure factors deposited in the Protein Data Bank, we have refined models with S at the FeMo-cofactor position of the putative N₂-derived species (6), and the refinement of S at these positions results in a high quality of fit based on residual electron density, with no remaining difference electron density at the belt sulfide positions and also without evidence for the reported switch of the homocitrate ligand from bidentate to monodentate (Fig. 1A). This refinement contradicts an assignment of a N₂-derived species with “high confidence.” Furthermore, at the reported resolution of 1.83 Å, the intensity signal-to-noise ratio I/σ(I) is 0.4, which is low, and it is important to point out that this resolution is low for making decisive statements about atomic species. For comparison, the data:parameter ratio (assuming isotropic B-factors) for Av1* (PDB 6UGO) is 0.85 at 1.83 Å resolution, whereas for the CO adduct of MoFe protein (PDB 4TKV) the ratio is 2.24 at 1.5 Å resolution, and for the turnover state of VFe protein (PDB 6FEA) it is 8.76 at 1.2 Å resolution.

The authors based their assignment of a N₂-derived species, rather than S, on purported changes in the anomalous scattering effect that occurs for an element near
the corresponding absorption edge. This assignment can be accomplished for S, but it is very difficult to collect data near the absorption edge of S because at this low energy (<3000 eV, λ = 4.1 Å), the diffraction quality of crystals is typically substantially reduced and the maximum achievable resolution is limited. The authors instead used data near 7000 eV for the analysis, which is not unusual, but one must be discriminating in the interpretation of such data. In the paper, for the assignment of a N2-derived species at S sites, the authors offer as support fluctuations in the S anomalous signals at three of the nine sulfur sites. These fluctuations of one or two sigma units, with reported averages of 5σ over all the sites, are not compelling, especially given the quality of the data (Fig. 1, B and C). Furthermore, the geometry and bond distances of the putative N2-derived species are unprecedented from literature N2 complexes (7). We argue that the most straightforward interpretation that fits all of the reported data is that the crystallographically characterized units have intact, native, unaltered, resting-state FeMo cofactors with nothing bound.

A piece of data offered in support of N2 binding is incubation of nitrogenase with 15N2 and subsequent quenching the enzyme with acid. This experiment led to release of a measurable, but unquantified, amount of 15N2 only in preparations isolated in the absence of the reductant dithionite, but not in its presence. The “reversibly bound species” was thus N2 and not a partially reduced N species, for which an acid treatment would be expected to yield hydrazine or ammonia (8), neither of which was reported.

REFERENCES

ACKNOWLEDGMENTS
Funding: Supported by NIH grants GM138592 (J.W.P.), GM111097 (B.M.H.), and GM-065313 (P.L.H.); U.S. Department of Energy, Office of Science, Basic Energy Sciences grants DE-SC00018143 (J.W.P.), DE-SC0010834 (D.R.D.), and DE-SC0010687 (L.C.S.); NSF grant MCB-1908587 (B.M.H.); Deutsche Forschungsgemeinschaft, PP 1927, project no. 311061829 (O.E.); and the Max Planck Society (S.D.) Author contributions: J.W.P. and O.E. coordinated the preparation of the narrative. Competing interests: The authors declare no competing financial or nonfinancial interests.
**Fig. 1. Assessment of nitrogen-to-sulfur replacement using original structure factors.** (A) Stereo view of a $2\text{F}_o - \text{F}_c$ map (blue mesh contoured at 1σ) and $\text{F}_o - \text{F}_c$ maps (positive green mesh and negative red mesh contoured at 3.5σ) calculated around FeMo-co in chain A after refinement with S replacing the previously modeled N$_2$ molecules. The S atoms are labeled S3A and S2B to reflect the naming for the native structure of the *Azotobacter vinelandii* MoFe protein structure. FeMo-co is presented in ball and sticks. Coloring scheme: carbon, green and gray; oxygen, red; nitrogen, blue; iron, gray; sulfur, yellow; molybdenum, dark red. (B and C) Spherical integration of the anomalous maps showing the spread of the S positions in the FeMo cofactor in chain A (B) and in chain C (C). Files related to re-refinement are available as a Zenodo archive (6). To assess the anomalous signal at the S positions of the two FeMo cofactors, we inspected an anomalous difference electron density map (“6UG0_Fig_2a_2d_Data_collected_at_7100eV”) deposited by the authors (9). The sulfur positions of the refined structure N2ase_Rib_refine_sulfur.pdb that contained μ$_2$-sulfides in all bridging positions were used, and electron density within the anomalous map was integrated over a sphere with $r = 0.7$ Å at these positions using the program MAPMAN (10). Re-refined coordinates for the MoFe nitrogenase structure were generated by refinement of 6UG0 structure with nitrogen atoms substituted for the sulfur atoms at the sulfur belt positions of FeMo-co using structure factors submitted with 6UG0. Refinement was performed using phenix.refine (11) (PHENIX-dev-3965 software suite) including reciprocal space, real-space, individual occupancy, and individual B-factor routines. Restraints published with the 6UG0 structure were used during the refinement: ICE and ICZ (FeMo-co), 1CL (P-cluster), HCA (homocitrate).
Comment on "Structural evidence for a dynamic metallocofactor during N₂ reduction by Mo-nitrogenase"

John W. Peters, Oliver Einsle, Dennis R. Dean, Serena DeBeer, Brian M. Hoffman, Patrick L. Holland and Lance C. Seefeldt

Science 371 (6530), eabe5481.
DOI: 10.1126/science.abe5481