Nitrogenases

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The Fe–V Cofactor of Vanadium Nitrogenase Contains an Interstitial Carbon Atom

*Julian A. Rees, Ragnar Bjornsson, Julia Schlesier, Daniel Sippel, Oliver Einsle, and Serena DeBeer**

Abstract: *The first direct evidence is provided for the presence of an interstitial carbide in the Fe–V cofactor of Azotobacter vinelandii vanadium nitrogenase. As for our identification of the central carbide in the Fe–Mo cofactor, we employed Fe Kβ valence-to-core X-ray emission spectroscopy and density functional theory calculations, and herein report the highly similar spectra of both variants of the cofactor-containing protein. The identification of an analogous carbide, and thus an atomically homologous active site in vanadium nitrogenase, highlights the importance and influence of both the interstitial carbide and the identity of the heteroatom on the electronic structure and catalytic activity of the enzyme.*

The biological fixation of atmospheric dinitrogen (N_2) to ammonium ions is exclusively promoted by nitrogenases (N_2ases), multicomponent metalloenzymes that occur in diazotrophic bacteria and archaea.[1,2] These enzymes utilize highly complex Fe–S clusters to effect N_2 reduction, and in the case of the more widely studied Mo-dependent N_2ase, the redox centers of the protein have been characterized by X-ray crystallography.[3] The active-site-containing MoFe protein, an $\alpha_2\beta_2$ heterotetramer, uses an [8Fe–7S] cluster (P-cluster) as an electron-transfer relay, and the [Mo–7Fe–9S–C] Fe–Mo cofactor (FeMoco) as the catalytic site of N_2 reduction (Figure 1). Despite the identification of an interstitial light atom at the center of FeMoco in 2002,[4] it was not until 2011 that the identity of the so-called "X" atom was definitively shown to be carbon by a combination of high-resolution X-ray crystallography, pulsed electron paramagnetic resonance (EPR) spectroscopy,[3] and Fe Kβ valence-to-core (VtC) X-ray emission spectroscopy (XES).[5]

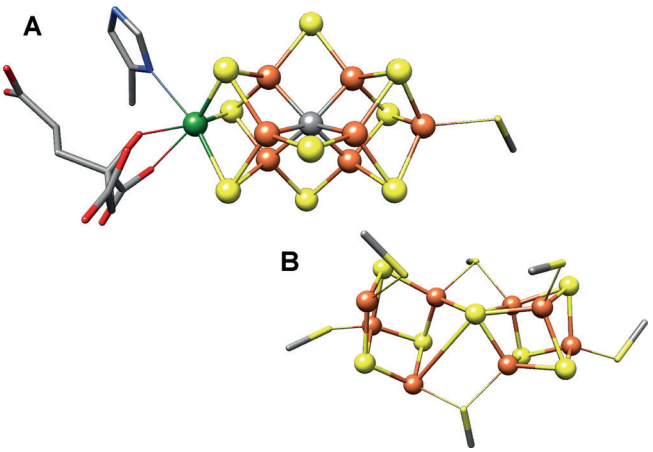

Figure 1. Representation of A) the FeMo cofactor and B) the P-cluster from *Azotobacter vinelandii* Mo nitrogenase, adapted from PDB 3U7Q. Color scheme for atoms: Fe = orange, S = yellow, C = gray, Mo = green, N = blue, O = red. Cysteinate residues are shown as sticks, and inorganic sulfides as spheres.

This fully valent carbide, ligated by six Fe atoms, is unprecedented and unique in biology. Genetic, spectroscopic, and isotopic radiolabeling experiments have determined the source of this carbon atom to be a radical *S*-adenosylmethionine (SAM), and have shown that the C atom is inserted into the cofactor through methyl group transfer in the cofactor assembly protein NifB.[6,7] The effect of this carbide ion on the electronic structure of the cofactor, and its role in promoting N_2 reduction, is still unknown.

Vanadium was first discovered as a promoter of nitrogen fixation in 1933,[8] and subsequently a V-dependent N_2ase was identified in 1986.[9] In contrast to the well-characterized Mo N_2ase, relatively little is known about the V N_2ase despite nearly 30 years of research. At ambient conditions the enzyme is a much poorer nitrogen fixation catalyst than the Mo analogue, capable of N_2 reduction at a rate of only 660 nmol mg^{-1} N_2ase min^{-1} compared to 1040 nmol mg^{-1} N_2ase min^{-1} for the Mo N_2ase in the *A. vinelandii* system.[10] Furthermore, the V N_2ase requires 4 more reducing equivalents, an additional 24 adenosine triphosphate (ATP) molecules per turnover, and has a significantly lower turnover number (112) than Mo N_2ase (2230).[11]

[*] J. A. Rees, Dr. R. Bjornsson,[+] Prof. Dr. S. DeBeer
Department of Molecular Theory and Spectroscopy
Max-Planck-Institut für Chemische Energiekonversion
Stiftstrasse 34–36, 45470 Mülheim an der Ruhr (Germany)
E-mail: serena.debeer@cec.mpg.de

J. Schlesier, D. Sippel, Prof. Dr. O. Einsle
Institut für Biochemie and BIOSS Centre for Biological Signalling Studies, Albert-Ludwigs-Universität Freiburg
Albertstrasse 21, 79104 Freiburg (Germany)

J. A. Rees
Department of Chemistry, University of Washington
Box 351700, Seattle, WA 98195-1700 (USA)

Prof. Dr. S. DeBeer
Department of Chemistry and Chemical Biology
Cornell University, Ithaca, NY 14853 (USA)

[+] Present address: Science Institute, University of Iceland
Dunhaga 3, 107 Reykjavik (Iceland)

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In 2010 it was shown that in addition to N_2 reduction, the V N_2ase can perform reductive C–C bond coupling using CO and protons.[12] Intriguingly, it also does so at roughly 700 times the activity of the native Mo N_2ase.[13] Engineered Mo N_2ases have been shown to catalyze CO reduction as well, and at rates comparable to V N_2ase, albeit with the loss of N_2 reduction ability.[14,15] The dramatic differences in the reactivity of these enzymes, and the ability of the vanadium enzyme to promote Haber–Bosch and Fischer–Tropsch chemistry at ambient conditions, has led to renewed interest in understanding the differences between the active-site cofactors in the Mo and V N_2ases.

The atomic structure of the V N_2ase protein is not known. Instead, the available structural information on the protein and its redox centers has come from spectroscopic and genetic studies. The latter have shown that while the two N_2ases share common precursors in their biosynthetic pathways, including the *nifB* gene responsible for radical SAM-dependent carbon insertion, distinct genetic factors promote final cofactor maturation.[16] Additionally, the V N_2ase protein contains additional δ_n subunits, where $n=2$ or 4 depending on the species.[17,18]

Much of the spectroscopic data collected on V N_2ase has also indicated substantial differences from Mo N_2ase. As early as 1987, Arber et al. and George et al. independently reported vanadium extended X-ray absorption fine structure (EXAFS) data indicating that the V atom, in contrast to expected changes based on periodic trends, was more displaced from the remainder of the cofactor than the Mo atom.[19,20] More recent studies employing Fe X-ray absorption spectroscopy (XAS), EXAFS, and EPR spectroscopy have also shown marked differences in the electronic structures of the V N_2ase metallocofactors. These differences are significant enough to prompt the authors to interpret their data as indicating the presence of a cubically symmetric Fe–V cofactor (FeVco) and two separated [4Fe–4S] clusters in place of the trigonal FeMo cofactor and fused P-cluster shown in Figure 1.[18,21–23] Despite this, some recent reports have shown graphical models of the redox centers of the V N_2ase as being structurally analogous to those of Mo N_2ase known from X-ray crystallography, in contradiction to the published data.[24,25] No direct evidence for an analogous cofactor structure has been presented in support of such models. This has led to the somewhat widespread belief that the structure of FeVco is identical to FeMoco, and that similarities in atomic composition and geometry are a foregone conclusion.

In an effort to provide more definitive evidence for the structure of FeVco, we report herein the Fe Kβ VtC XES spectrum of the VFe protein of *A. vinelandii* N_2ase (Figure 2). We have also concomitantly re-measured the analogous spectrum of the MoFe protein, and note that the Kβ'' feature at approximately 7100 eV, previously shown to be indicative of fluorescent emission from the 2s atomic orbital of the central carbide in FeMoco, is identically placed in the spectrum of VFe. Additionally, the difference spectrum shows only minute deviations in the intensities and energies in all spectral regions.

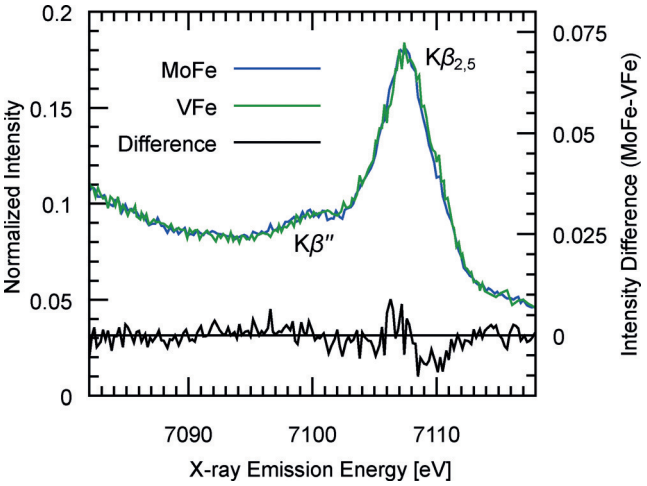

Figure 2. Fe Kβ VtC XES spectra of MoFe and VFe proteins from *A. vinelandii*. The Kβ'' peak at 7099.8 eV has been previously shown to be attributable to the interstitial carbon in the FeMo cofactor of MoFe. The absence of an intensity difference (MoFe −VFe) at the Kβ'' energy indicates an interstitial carbon is also present in FeVco.

We have previously shown that the energy of the distinctive Kβ'' peak in FeMoco is inconsistent with any interstitial light atom besides carbon, and that in the absence of such a carbon atom, for example in the P-cluster, the intensity of the Kβ'' feature is significantly diminished.[5] Given the virtually identical VtC spectra of the MoFe and VFe proteins, we believe these data strongly indicate the presence of an analogous interstitial carbide in FeVco, thereby providing, to our knowledge, the first direct evidence for a structurally homologous cofactor in the V N_2ase.

To provide additional support for this conclusion, we have performed density functional theory (DFT) calculations on large, 225-atom models of both cofactors, based on the published crystal structure of MoFe. The FeMoco model was previously utilized in our report on the determination of the Mo^{III} oxidation state in FeMoco,[26] and the FeVco model was calculated as being isostructural and valence isoelectronic (see the Experimental Section for details). Our optimized structure of the FeVco active-site model results in V–Fe distances that are 0.11 Å longer than the Mo–Fe distances in the optimized structure of FeMoco, in reasonably good agreement with experimental metrical parameters from EXAFS (0.08 Å longer).[20] VtC XES spectra were calculated within a one-electron approximation, and the averages of the spectral contributions from all cofactor Fe atoms are presented in Figure 3.

The calculations, like the experimental results, show strikingly similar spectra in both the K$\beta_{2,5}$ and Kβ'' regions, where the latter is dominated by S 3s and C 2s contributions and the former by S 3p contributions. The subtle differences in the calculated spectra are highlighted by a difference spectrum, which again agrees well with experiment. In particular, the derivative shape under the K$\beta_{2,5}$ peak is reproduced by the calculations and may be attributed to the sulfur 3p orbitals being shifted to slightly higher energy in FeVco than in FeMoco. We caution, however, that because of

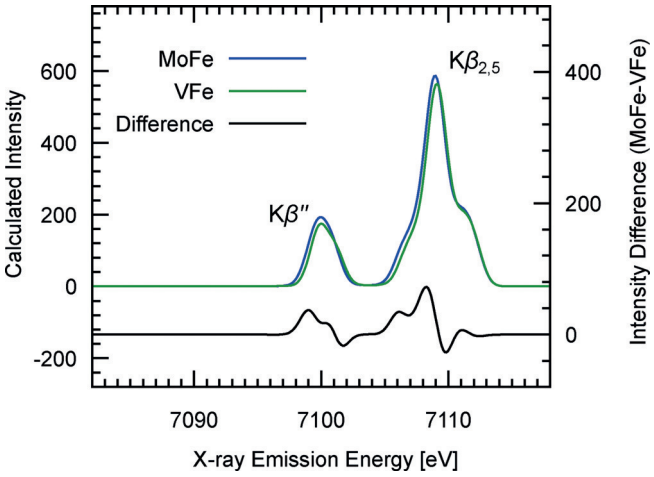

Figure 3. DFT-calculated Fe Kβ VtC XES spectra of 225-atom models of FeMoco and FeVco. Spectra are an average of calculated transitions from all seven Fe atoms, and a 1.5 eV broadening was applied to discrete transition moments. Features in the difference spectrum (MoFe−VFe) correspond well to the experimental data. A scalar shift of 123 eV was applied to correct the absolute transition energies.[27]

the limited experimental resolution, a more quantitative analysis is not possible. Nonetheless, these spectra clearly establish the presence of a carbide in FeVco. A more detailed study of the electronic structural differences will likely require higher resolution spectroscopies (such as high-resolution (HERFD) XAS)[28] and will be the topic of future studies in our laboratories.

The present work takes an initial step toward directly defining the structure of FeVco. The presence of a second biological cofactor with an interstitial carbide has now been experimentally established. With this finding, we have laid the groundwork for more focused studies aimed at understanding how perturbations to electronic structure, likely engendered by the heterometal, differentially tune these remarkable enzymes, enabling reactions as diverse as N_2 activation and C–C bond coupling under ambient conditions.

Experimental Section

Cell growth and protein purification: MoFe protein of N_2ase was produced and isolated following established procedures.[3] To obtain the VFe protein, *Azotobacter vinelandii* (Lipmann 1903, ATCC 478) was cultured in molybdenum-free Burke medium[29] under nitrogen-limited conditions. The production of vanadium N_2ase was monitored by activity assays.[30] All purification steps were performed under strict exclusion of dioxygen, using an anaerobic chamber or modified Schlenk techniques. Cells were disrupted using an Avestin Emusiflex at a pressure of 1000–1500 bar, and cell debris was separated by centrifugation. The supernatant was loaded onto a 5 mL HiTrapQ HP column equilibrated with 50 mM Tris/HCl buffer at pH 7.4. VFe protein eluted in a linear gradient of 0 mM to 500 mM NaCl. Pure protein was obtained after an additional size-exclusion step on a 26/60 Superdex 200 gel filtration column (GE Healthcare).

X-ray spectroscopy: Fe Kβ X-ray emission spectroscopy (XES) experiments were performed at beamline ID-26 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Incident photon energy was 7800 eV, selected using a Si(111) double crystal monochromator. Photon flux at the sample was approximately 1×10^{13} photons sec^{-1}, with a maximum ring current of 200 mA and a ring energy of 6.03 GeV. The beam spot size on the sample was 0.1 mm × 1 mm. Protein samples were in aqueous solution at approximately 100 mg mL^{-1}, loaded in Delrin cells sealed with 38 μm Kapton tape and maintained at 10 K during measurements using a liquid He cryostat. Fe Kβ X-ray emission was analyzed with a Johann-type spectrometer, using five spherically bent Ge(620) crystals in a Rowland geometry, as described previously,[31] and detected using a dead-time-corrected Ketek Si drift diode detector. To determine the acceptable dwell time per sample spot, rapid Fe Kβ high-energy resolution fluorescence detected (HERFD) X-ray absorption spectra were recorded on the same sample spot, and the data examined for evidence of change during the course of sample dosing. For all XES measurements, the photon dose was well below the acceptable limit. Multiple scans on the same sample were normalized to incident flux and averaged using MATLAB. Data were referenced to the K$\beta_{1,3}$ and K$\beta_{2,5}$ features of Fe_2O_3[27] and normalized to a total integrated spectral intensity of 100 by numerical integration.

Density functional theory calculations: All calculations were performed using the ORCA program package developed by Neese and co-workers.[32] 225 atom cluster models of FeMoco and FeVco active sites were based on the X-ray structure of MoFe protein,[3] and were TPSSh-optimized using our previously reported procedure.[26] Charges on the metal clusters were −1 for FeMoco ($[MoFe_7S_9C]^{1-}$) and −2 for FeVco ($[VFe_7S_9C]^{2-}$), to maintain a valence isoelectronic configuration and a spin of $S=3/2$. We note that other cofactor charges are conceivable, however our previous studies have shown that VtC spectra are relatively insensitive to changes in cofactor charge,[5] and thus we have not considered them here. Analogous broken-symmetry solutions were found. Valence-to-core XES spectra were calculated within a one-electron approximation implemented in ORCA as described in Ref. [27]. These calculations used the BP86 functional[33,34] and the DKH relativistic approximation,[35–37] with DKH-recontracted def2-TZVP triple-zeta basis sets[38,39] and the COSMO dielectric model ($\varepsilon=4$).[40]

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