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X-ray Spectroscopic Observation of an Interstitial Carbide in NifEN-Bound FeMoco Precursor

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Supporting Information

ABSTRACT: The iron−molybdenum cofactor (FeMoco) of nitrogenase contains a biologically unprecedented μ^6-coordinated C^{4-} ion. Although the role of this interstitial atom in nitrogenase catalysis is unknown, progress in understanding its biosynthetic origins has been made. Here we report valence-to-core Fe Kβ X-ray emission spectroscopy data to show that this C^{4-} ion is present in the Fe_8S_9 "L-cluster," which is the immediate precursor to FeMoco prior to the insertion of molybdenum and coordination by homocitrate. These results accord with recent evidence supporting a role for the *S*-adenosylmethionine-dependent enzyme NifB in the incorporation of carbon into the FeMoco center of nitrogenase.

The iron−molybdenum cofactor (FeMoco) of nitrogenase is nature's most active catalyst for the conversion of inert N_2 to bioavailable NH_4^+.[1,2] A complex metallocluster with a Fe_7S_9Mo core, the FeMoco is coordinated in the host protein by homocitrate and a histidine at the Mo end and a cysteine at the opposite Fe end (Figure 1b). It also contains an interstitial light atom that was identified recently as a carbide (C^{4-}) ion.[3,4] The role of the interstitial C^{4-} is unclear, and to date, no synthetic models have emerged to assist in the elucidation of its contribution to nitrogenase activity. Unraveling when and how this atom is incorporated into the cofactor structure will guide studies of its function.

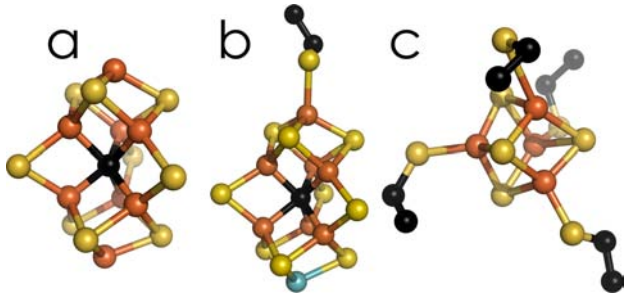

Figure 1. Core structures of (a) the precursor in NifEN (PDB entry 3PDI) and (b) the FeMoco in MoFe protein (PDB entry 3U7Q). The interstitial C^{4-} ion is included in both structures. (c) Structure of the O-cluster in NifEN. Atoms are colored as follows: S, yellow; Fe, orange; C, black; Mo, cyan.

Biosynthesis of FeMoco starts with the formation of Fe_2S_2 and Fe_4S_4 fragments on NifS and NifU, which is followed by the formation of a precursor from these small FeS fragments on NifB. This precursor, the "L-cluster" (Figure 1b), is transferred to NifEN.[5] Maturation to the catalytically active FeMoco is then achieved by metal substitution of an apical Fe with Mo and coordination of homocitrate.

A recent report has shown that ^{14}C can be traced from the *S*-methyl moiety of *S*-adenosylmethionine (SAM) to NifB, then to NifEN, and ultimately to the active MoFe protein.[6] Multiple lines of evidence indicate that the couriers of this radiolabel are the L-cluster and FeMoco, clearly demonstrating that the C^{4-} in FeMoco originates from SAM. However, incomplete structural characterization has left the mode of association of the ^{14}C with the L-cluster unclear.

The structure of the L-cluster was initially described as either a seven or eight Fe core on the basis of extended X-ray absorption fine structure (EXAFS) studies. Subsequent EXAFS studies of the isolated L-cluster established an eight Fe core and identified a light-atom contribution that could be attributed to either the interstitial atom or solvent.[7] Crystallographic analysis provided further support for an Fe_8S_9 model of this cluster, although the 2.6 Å resolution structure also could not unambiguously indicate the presence of an interstitial light atom.[8] Moreover, structural data at this resolution could not dismiss the possibility that one of the μ^2-S donors is methylated. We note that EXAFS and nuclear resonance vibrational spectroscopy (NRVS) have been used to argue for the presence of an interstitial light atom in NifB-co, a proposed FeMoco precursor.[9−11] However, NifB-co is likely irrelevant to FeMoco biosynthesis, particularly in light of recent results showing that the L-cluster can be directly assembled from Fe_4S_4 clusters without proceeding through NifB-co.[12] We thus chose to focus our studies on NifEN, which binds the immediate precursor to FeMoco, thus allowing us to deduce temporally the point of carbon insertion.

Valence-to-core (V2C) Fe Kβ X-ray emission spectroscopy (XES) is a powerful technique for probing the identity of inner-sphere ligands[3,13−18] that does not require crystalline samples

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or isotopic enrichment. V2C XES measures the spectrum of photons emitted when electrons populating ligand-centered molecular orbitals (MOs) are demoted energetically to fill an Fe 1s core hole generated by X-ray induced photoionization. V2C XES features arising due to transitions from MOs of predominantly ligand *n*s parentage are called Kβ'' transitions. Kβ'' transitions serve as elemental fingerprints because the MOs involved minimally participate in chemical bonding and thus are primarily sensitive to atomic ionization energies. Light-atom Kβ'' bands are well-separated, occurring near 7092, 7096, and 7100 eV for O, N, and C, respectively.[3,15] Moreover, the requirement of mixing and therefore overlap with Fe 4p orbitals to confer intensity to these transitions means that only inner-sphere atoms contribute.[16,17] Additionally, V2C XES can differentiate charged states of inner-sphere donors. For example, the N 2s V2C bands of N^{3-}, HN^{2-}, and H_2N^- are predicted to be shifted by 1 eV per protonation.[18] The utility of this method renders it the most suitable approach for ascertaining the presence of an interstitial atom in the NifEN-bound L-cluster.

Here we report the V2C XES of NifEN (containing the Fe_8S_9 L-cluster and the Fe_4S_4 "O-cluster") and Δ*nifB* NifEN (containing only the O-cluster). The Δ*nifB* variants of *Azotobacter vinelandii* do not produce NifB, which is required for conversion of the Fe_4S_4 clusters to the Fe_8S_9 L-cluster on NifB. Consequently, FeMoco biosynthesis is stalled at a step prior to the sequential formation of the L-cluster and FeMoco, resulting in the production of Δ*nifB* NifEN (L-cluster-deficient) and Δ*nifB* MoFe (FeMoco-deficient) proteins, which can be used for the analysis of background Fe−S contributions to V2C XES data. Our present results mark a more rigorous spectroscopic deconvolution of interstitial atom contributions to V2C XES than the removal of an empirical S^{2-} contribution as we reported previously.[3] However, the same net result is achieved.

We directly subtracted the V2C XES data for Δ*nifB* NifEN from those for extracted FeMoco, which permitted an unbiased correction for background X-ray fluorescence. Since Δ*nifB* NifEN binds only the Fe_4S_4 O-cluster, subtraction of its spectrum also allowed us to remove the S^{2-} contributions from the V2C data in correct proportion to the number of emissive Fe atoms. RS^- contributions to the spectra are expected to be insignificant relative to S^{2-}, as we have previously demonstrated.[3] Thus, any intensity arising from an interstitial species can be isolated in this manner. Indeed, this procedure revealed significant residual intensity in FeMoco at 7099.8 ± 0.1 eV (Figure 2a, gray) that is consistent with the previously reported value of the interstitial C^{4-} in FeMoco.[3] Likewise, subtraction of the V2C data for Δ*nifB* NifEN from those for NifEN revealed residual intensity at 7099.7 ± 0.4 eV (Figure 2b, red) that is indicative of the presence of interstitial C^{4-} in the precursor. Subtraction of NifEN from FeMoco removed all intensity from the 7090−7103 eV region (Figure 2b, blue), providing further confirmation of the presence of a C^{4-} in both clusters.

For better comparison of the FeMoco and the L-cluster in their native protein environments with the isolated FeMoco, we also performed subtractions of V2C data for Δ*nifB* MoFe protein and Δ*nifB* NifEN from those for MoFe protein and NifEN, respectively, thus removing the corresponding P-cluster and O-cluster contributions to the spectra (Figure 3). While these spectra still possess the 2s feature of C^{4-} at 7100 eV, they highlight differences in the higher-energy regions of the spectra.

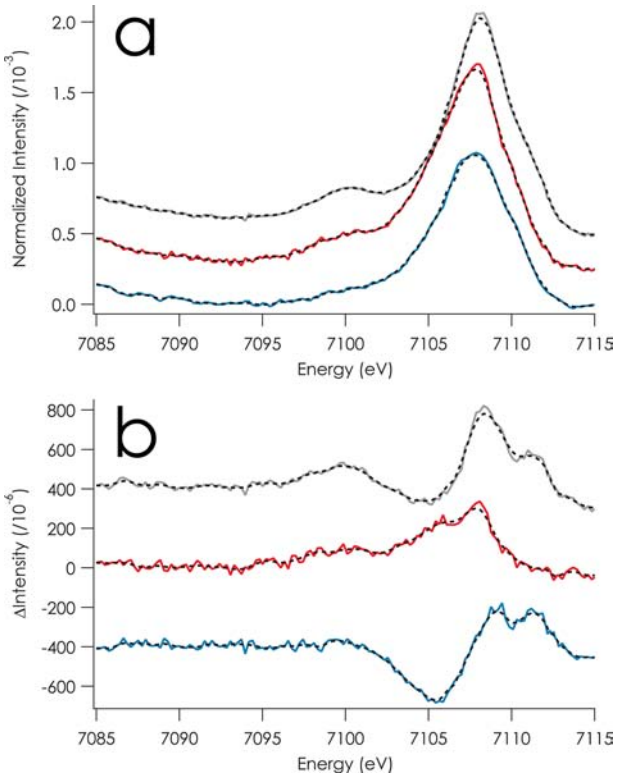

Figure 2. (a) V2C XES spectra of extracted FeMoco (gray), NifEN (red), and Δ*nifB* NifEN protein (blue). (b) Difference spectra between extracted FeMoco and Δ*nifB* NifEN (gray), NifEN and Δ*nifB* NifEN (red), and extracted FeMoco and NifEN (blue). Smoothed spectra are overlaid in dashed black lines for clarity.

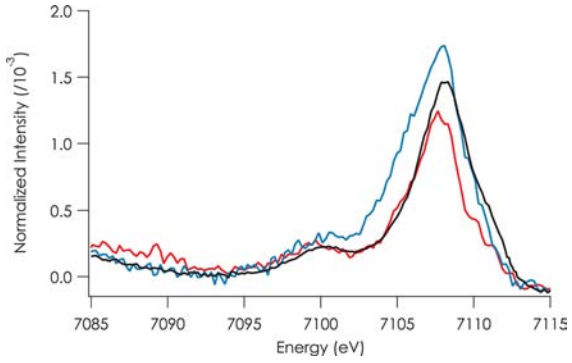

Figure 3. V2C XES spectra of isolated FeMoco (black), MoFe protein corrected for P-cluster contributions (red), and NifEN protein corrected for O-cluster contributions (blue).

In particular, deconvolution of contributions to the spectral intensity in the 7103−7115 eV region, which comprises V2C transitions from occupied MOs of ligand *n*p parentage, is complicated by substantial metal−ligand MO mixing. Regardless, data derived from these subtractions indicate a perturbation of the environment of the L-cluster relative to those of the native and extracted FeMoco. We propose that the greater intensity near 7105 eV exhibited by the NifEN-bound L-cluster could originate from the binding of H_2O to exposed Fe sites, resulting in an O 2p contribution to the XES spectrum at ∼7105 eV.[14,15] We also suggest that the differences between the spectra of isolated FeMoco in *N*-methylformamide (NMF) and native FeMoco within the MoFe protein environment may reflect the impact of NMF binding on the electronic structure

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of the cofactor. Specifically, replacement of cysteine by NMF in extracted FeMoco would replace some S 3p contributions to this region with O/N 2p contributions.

In summary, we have employed V2C XES to establish the presence of the interstitial C^{4-} in the Fe_8S_9 L-cluster, which is the immediate precursor to FeMoco prior to the insertion of molybdenum and homocitrate. Our results indicate that at this stage of the biosynthesis the carbon species is fully deprotonated and bound to the iron. Our observation of a NifEN-associated Fe_8S_9C cluster is consistent with C^{4-} incorporation via a NifB-catalyzed, radical SAM-dependent mechanism.[12] The exact mechanism of such a process, as well as its adaptation to synthetic model chemistry, merits further investigation.

■ ASSOCIATED CONTENT

Supporting Information

Methods for sample preparation and XES measurements and analysis, full Kß XES spectra for all samples, and fits to difference spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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