Published in final edited form as:

Chem Commun (Camb). 2016 September 27; 52(79): 11811–11814. doi:10.1039/c6cc06370e.

EXAFS reveals two Mo environments in the nitrogenase iron-molybdenum cofactor biosynthetic protein NifQ[†]

Simon J. George^{‡,a}, Jose A. Hernandez^{‡,b}, Emilio Jimenez-Vicente^c, Carlos Echavarri-Erasun^c, and Luis M. Rubio^c

Luis M. Rubio: lm.rubio@upm.es

^aSimon Scientific Inc., Berkeley, California 94701

^bDepartment of Biochemistry, Midwestern University, Glendale, Arizona 85308

^cCentro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM) – Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). Campus Montegancedo UPM, 28223-Pozuelo de Alarcón. Madrid (Spain)

Abstract

Mo and Fe K-edge EXAFS analysis of NifQ shows the presence of a [MoFe₃S₄] cluster and a second independent Mo environment that includes Mo-O bonds and Mo-S bonds. Both environments are relevant to FeMo-co biosynthesis and may represent different stages of Mo biochemical transformations catalyzed by NifQ.

Graphical abstract

Two molybdenum environments in the iron-molybdenum cofactor biosynthetic protein NifQ

Molybdenum is the only second-row transition metal essential for nearly all living organisms. It is present in the active sites of over 50 different molybdoenzymes, some of which catalyze key reactions in global carbon, nitrogen, or sulfur cycles.¹

The [Fe₇-S₉-Mo-C-homocitrate] iron-molybdenum cofactor (FeMo-co), present at the active site of the Mo nitrogenase, is essential to catalyze the reduction of N₂ into ammonia.² Due to

[†]Electronic Supplementary Information (ESI) available: Experimental and spectroscopic details. See DOI: 10.1039/x0xx00000x

Correspondence to: Luis M. Rubio, lm.rubio@upm.es.

[‡]These authors contributed equally.

its structural complexity FeMo-co is biosynthesized by a complex pathway that involves the activity of many nitrogen fixation (*nif*) gene products.³ FeMo-co is composed of a [Fe₆S₉-C] cluster capped by an Fe atom on one end and by a Mo atom on the opposite end, with the Mo atom additionally coordinated by the C-2 carboxyl and hydroxyl groups of *R*-homocitrate.⁴ Whereas the origin of Fe and S of FeMo-co has been ascribed to NifB,⁵ the biochemistry required to transform MoO₄²⁻ into the Mo atom of FeMo-co is unknown.

NifQ is a protein involved in the incorporation of Mo into FeMo-co.^{6–8} Purified NifQ samples contain an average of 3.1 Fe and 0.30 Mo atoms per NifQ. Biochemical and electron paramagnetic resonance (EPR) spectroscopic evidence has suggested that NifQ coordinates Mo in a [MoFe₃S₄] cluster and serves as Mo donor to the NifEN/NifH protein complex, which catalyzes Mo and homocitrate incorporation into FeMo-co.⁶

Here we use Mo and Fe K-edge X-ray absorption spectroscopy (XAS) to study the ligand environment and the oxidation state of the Mo and Fe atoms of NifQ. Interpretation of these data permits a better understanding of the speciation of Mo in NifQ, and thus its role FeMo-co biosynthesis.

Fig. 1 compares the Mo K-edge near-edge spectrum of NifQ with data from a Na₂MoO₄ solution and proteins containing MoFeS clusters: NifEN,⁹ the nitrogenase MoFe protein¹⁰ and the carrier protein NafY bound to FeMo-co (NafY:FeMo-co).¹⁰ Integration of Mo K α fluorescence at 20.5 keV incident energy rendered 1.7 mM Mo for 5 mM NifQ.[§] The NifQ near-edge spectrum is similar in structure and energy to those of NifEN and the FeMo-co containing proteins, and quite distinct from Na₂MoO₄. This suggests that the Mo in NifQ has a similar ligand field and oxidation state to these proteins, namely Mo(IV), in a mixed S, O ligand environment and possibly part of a MoFeS cluster. However, we cannot exclude the possibility of some Mo being Mo(V) or Mo(VI). The NifQ spectrum shows a distinct shoulder at ~ 20.008 keV, which is also present in NifEN and NafY:FeMo-co, albeit much less pronounced. This pre-edge feature is similar to the oxo-edge band characteristic of species possessing Mo=O groups, or to a lesser extent Mo=S.¹¹ It arises from formally forbidden Mo 1s $\rightarrow$ Mo=O (or Mo=S) π^* transitions directed along the Mo=O(S) bonds.¹² This band is evident in the Na₂MoO₄ solution (Fig. 1e).¹³

Fig. 2 shows the Fe and Mo extended X-ray absorption fine structure (EXAFS) spectra and Fourier transforms, while fitting parameters are presented in Table 1. The Fe EXAFS (Fig. 2a and b) is best modeled using a [Fe₃S₄] cluster with 2 Fe atoms at a distance of 2.71 Å and 4 S atoms at 2.27 Å. It is possible to include a small Fe-Mo contribution corresponding to the Mo-Fe interaction at 2.71 Å observed in the Mo EXAFS (see below). However, as this Fe-Mo interaction is essentially out of phase with the observed Fe-Fe interaction, including it does not significantly change the Fe-S or Fe-Fe distances or improve the fit quality. Hence, as it is not required for an *a priori* good fit, and it does not provide any new useful information, this Fe-Mo interaction is not included in Fig 2a or Table 1.^{§§}

[§]Examination of the X-ray fluorescence spectrum confirmed that NifQ contains Mo and Fe and no significant quantities of other metals. Quantitation of the Mo fluorescence intensity was consistent with reported Mo occupancy of 0.3 atoms per NifQ.

^{§§}The effect of including a Fe-Mo interaction is discussed in detail in the ESI.

The Mo K-edge EXAFS (Fig. 2c and d) reveals the presence of a MoFeS cluster with Mo-S distance of 2.34 Å and Mo-Fe distance of 2.71 Å. The Fourier transform shows at least 2 additional interactions, including a short Mo-O interaction at 1.73 Å and a long Mo-S interaction at 2.23 Å. This makes NifQ a more complex Mo system than, for example, NifEN.⁹ In analyzing these data, several models were trialed. While Fig. 2 presents the best fit, full details of all the fits are in the ESI[†].

The first trialed model combined the Mo-Fe, Mo-S and Mo-O interactions into a single [MoFe₃S₄] cluster with a novel short Mo=O bond. However, it proved not possible to achieve a good fit without assuming a highly distorted cluster. A second model assumed that 27% of the Mo was present as MoO₄²⁻, which would account for the presence and size of the pre-edge feature at 20.008 keV, and with the [MoFe₃S₄] cluster comprising the remaining 73% Mo. However, not only did this model not give a good fit, but the observed Mo-O bond distance of 1.73 Å is significantly shorter than both the 1.79 Å of MoO₄²⁻ free in solution and the 1.75–1.77 Å of MoO₄²⁻ bound to molbindin proteins.¹³ In addition, XAS inspection of the aqueous phase in NifQ preparations showed no sign of free MoO₄²⁻. The most successful models assumed two Mo environments, each containing approximately half the total Mo. The best fit, with the most reasonable Debye-Waller factor for the 2.34 Å Mo-S interaction, was achieved by including an additional short Mo-S at 2.23 Å.^{§§§} Including longer Mo-O bonds at around 2.12 Å consistently improved the fit, although in Fig. 2 and Table 1 these contribute only 3% intensity to the overall EXAFS.

Hence, the EXAFS analyses strongly suggest that NifQ contains a [MoFe₃S₄] cluster, which likely includes Mo-O at 2.12 Å, and accommodates about half the bound Mo. In addition, the Fe K-edge near-edge spectrum (Fig. 3) and the Fe EXAFS are consistent with the presence of [Fe₃S₄] clusters in NifQ. This agrees with previous metal and EPR analyses, which suggested that each NifQ molecule carries either a [Fe₃S₄] cluster or a [MoFe₃S₄] cluster.⁶ The best Mo EXAFS fits suggest that a second Mo environment is also present, including a short 1.73 Å Mo-O and possible Mo-S bonds at 2.23 Å. The existence of this second Mo form in NifQ may explain the observation that only 50% of the Mo is transferred from native NifQ to NifEN/NifH under FeMo-co synthesis conditions.⁶

In an experiment designed to alter the ratio of its Mo environments, NifQ was incubated either with α, α'-bipyridyl or CuCl₂. The resulting NifQ proteins were then analyzed by EXAFS and by their ability to serve a Mo donor for FeMo-co synthesis *in vitro*. Mo K-edge EXAFS data are presented in Fig. 4, fitting parameters are presented in Table S3 of ESI[†], and Mo donor activity measurements are in Table 2.

Mo EXAFS of the product of a control reaction in which NifQ was incubated with assay buffer (see ESI[†] for experimental procedures) showed slightly different ratios of Mo=O content compared to as-isolated NifQ (compare Fig. 4a and b). Mo EXAFS of the α, α'-bipyridyl treated sample was consistent with α, α'-bipyridyl chelating a fraction of the Fe present in the [MoFe₃S₄] cluster without largely altering this Mo environment (Fig. 4c). On

[†]Electronic Supplementary Information (ESI) available: Experimental and spectroscopic details. See DOI: 10.1039/x0xx00000x

^{§§§}This is separated from the 2.34 Å Mo-S by more than the calculated EXAFS resolution of 0.093 Å.

the other hand, treatment with CuCl_2 practically eliminated the second Mo environment, as indicated by the increase in the number of 2.26 Å Mo-S and 2.69 Å Mo-Fe bonds and the decrease of the 1.73 Å Mo-O bonds (Fig. 4d and Table S3). This CuCl_2 -treated NifQ sample exhibited a Cu environment dominated by a single broad peak that fitted well assuming 2 Cu-O and 2 Cu-S interactions (Fig. S2 and Table S4 of ESI[†]). Importantly, there was no evidence of Cu-Mo interactions at 2.7 Å, confirming that the observed interactions are indeed Mo-Fe.

Upon treatment with either α , α' -bipyridyl or CuCl_2 , NifQ ability to serve as Mo donor in the *in vitro* FeMo-co synthesis and insertion assay was also investigated (Table 2). The Cu(II) treatment almost completely abolished NifQ ability to serve as Mo donor for FeMo-co synthesis while the α , α' -bipyridyl treatment decreased it by 34%. Thus, both Mo environments within NifQ are relevant to its function as Mo donor. The relative strength of the inactivating treatments might be due to relative accessibility of metals to Cu(II) and α , α' -bipyridyl.

The low Mo occupancy in NifQ preparations (~ 0.3 Mo per NifQ) was discussed in detail in Hernandez *et al.*⁶ and can be interpreted as these preparations reflecting a snapshot of a dynamic system with input and output of Mo from NifQ during FeMo-co biosynthesis. The current data does not allow to distinguish the possibility of heterogeneous NifQ (i.e. all NifQ molecules having only one site, which in some of them is occupied by a $[\text{MoFe}_3\text{S}_4]$ cluster and in others by, for example, oxothiomolybdate) from that of a single NifQ molecule with multiple Mo environments (Fig. S3). Nevertheless, the presence of two apparent Mo binding environments in NifQ is relevant to the Mo biochemistry for FeMo-co biosynthesis. NifQ is known to donate Mo to the NifEN/NifH biosynthetic machinery to complete FeMo-co.^{6,14} Mo is initially imported into the cell as MoO_4^{2-} and the subsequent conversion of this Mo(VI) oxyanion into the reduced Mo(IV) present in FeMo-co requires three chemical events: replacement of oxo-ligands by S ligands, reduction of Mo, and insertion of Mo into an $[\text{Fe-S}]$ cluster environment.

The $[\text{Fe}_3\text{S}_4]$ cluster observed in isolated NifQ molecules is an obvious site for Mo binding, reduction and subsequent transfer. This is not just because a fraction of the $[\text{Fe-S}]$ clusters in NifQ were found to be $[\text{MoFe}_3\text{S}_4]$ centers, but also because the reduced $[\text{Fe}_3\text{S}_4]^0$ state is known to be able to coordinate heterometals to complete $[\text{MFe}_3\text{S}_4]$ cubanes in reversible equilibrium that can be gated by the cluster oxidation state.^{15,16} This implies that the $[\text{Fe}_3\text{S}_4]$ cluster could be capable of binding and reducing Mo, releasing it in response to an oxidative trigger. However, a necessary preliminary step would be the partial or total replacement of MoO_4^{2-} oxo-ligands by S. The observation in NifQ of a separate Mo environment with an apparent mixture of Mo=O and short Mo-S bonds suggests this second site may be the locus for this Mo binding and subsequent S substitution in a mechanism that is unclear.

An alternative hypothesis for the role of the second environment is that it represents the form of Mo that is delivered to NifEN/NifH for FeMo-cofactor biosynthesis. The fact that removal of this site by Cu(II) treatment completely eliminates NifQ ability to support nitrogenase activation would be consistent with this role.

In conclusion, EXAFS analyses confirm that NifQ can contain a [MoFe₃S₄] cluster. A second Mo environment is apparent which may comprise a separate binding site. These data strongly suggest that rather than solely acting as scaffold, NifQ may have an enzymatic role in processing MoO₄²⁻ into a form capable of efficient transfer to FeMo-co.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Stephen Cramer for discussions and support. We also thank Christopher J. Pickett for helpful discussions. This work was supported by ERC Starting Grant 205442 (L.M.R.), MINECO grant BIO2014-59131-R (L.M.R.), Midwestern University intramural funds (J.A.H.) and GM-65440 to Stephen P. Cramer. Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL), a national user facility operated by Stanford University on behalf of the DOE OBES. The SSRL Structural Molecular Biology Program is supported by the DOE OBER, and the NIH.

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Fig. 1. Mo K-edge near-edge spectra (left panel) and first derivative spectra (right panel) from (a) *A. vinelandii* NifQ, (b) *A. vinelandii* NifEN, (c) NafY:FeMo-co complex, (d) *A. vinelandii* MoFe protein, and (e) aqueous Na₂MoO₄ solution.

Fig. 2.

Mo and Fe K-edge EXAFS from *A. vinelandii* NifQ. Data and fits are presented as broken and solid lines, respectively. (a) k^3 -weighted Fe EXAFS spectrum and (b) Fe-S phase corrected Fourier transforms. (c) k^3 -weighted Mo EXAFS spectrum and (d) Mo-S phase corrected Fourier transforms.

Fig. 3. Fe K-edge near-edge spectrum recorded from *A. vinelandii* NifQ (left panel) together with its first derivative (right panel). The spectrum is typical of an Fe-S protein.

Fig. 4. Mo K-edge EXAFS of NifQ. (a) As-isolated NifQ. (b) NifQ after buffer-exchange treatment. (c) NifQ after α, α' -bipyridyl treatment. (d) NifQ after CuCl_2 treatment. Data and fits are presented as broken and solid lines, respectively. Left panels, k^3 weighted Mo EXAFS spectra. Right panels, Mo-S phase corrected Fourier transforms from left panels.

Table 1

Fitting parameters for Mo and Fe EXAFS.

Interaction	N	R (Å)	σ^2 (Å ²)	E ₀ (eV)
Mo-S (long)	1.5	2.336 (6)	0.0040 (8)	
Mo-Fe	1.5	2.713 (2)	0.0034 (1)	
Mo-O (short)	0.9 (1)	1.734 (2)	0.0019 (5)	−9.7 (6)
Mo-S (short)	0.9 (1)	2.227 (17)	0.0038 (21)	
Mo-O (long)	1.1 (6)	2.124 (28)	0.0050	
Fe-S	4.0	2.269 (1)	0.0037 (1)	
Fe-Fe	2.0	2.707 (2)	0.0048 (2)	−13.3 (4)

N is the number of atoms, R the distance, σ^2 the Debye-Waller factor, and E₀ the offset from the threshold energy. The parameters that were allowed to float have fitting uncertainties to the indicated precision in brackets.

Table 2

Effect of selectively altering Mo environments in NifQ on its ability to support *in vitro* FeMo-co synthesis.

Sample	Nitrogenase Activity
NifQ	
after buffer-exchange treatment	50.2 ± 2.8
after α , α' -bipyridyl treatment	32.9 ± 8.1
after CuCl ₂ treatment	1.9 ± 0.1
MoO ₄ ²⁻	36.4 ± 5.3
Without Mo source	2.0 ± 0.1

In vitro FeMo-co synthesis and nitrogenase activity assays were performed with purified components. Reaction mixtures included a large excess of apo-NifDK. After FeMo-co synthesis, the amount of reconstituted NifDK is determined by the acetylene reduction assay. Nitrogenase specific activity is given in nmol C₂H₄·min⁻¹·mg⁻¹ NifDK. Data is the mean of at least two independent determinations ± standard deviation.