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Is Mo Involved in Hydride Binding by the Four-Electron Reduced (E_4) Intermediate of the Nitrogenase MoFe Protein?

Dmitriy Lukoyanov,[†] Zhi-Yong Yang,[§] Dennis R. Dean,*[,‡] Lance C. Seefeldt,*[,§] and Brian M. Hoffman*[,†]

Department of Chemistry, Northwestern University, Evanston, Illinois 60208, Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061, and Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

Received December 16, 2009; E-mail: bmh@northwestern.edu

The X-ray structure[1] of the nitrogenase[2] molydenum-iron (MoFe) protein reveals the active-site FeMo-cofactor (FeMo-co) to be an unprecedented [Fe_7S_9MoX; homocitrate] cluster, Figure 1, but does not define the location of substrate binding and reduction. Mo is an obvious candidate, as it is the catalytic metal in the only known inorganic metal complexes that catalytically reduce N_2.[3] However, Fe is no less a candidate, given that it is the catalytic metal in the commercial Haber−Bosch process for NH_3 formation and that there are V and Fe nitrogenases that reduce N_2 but do not have Mo.[4]

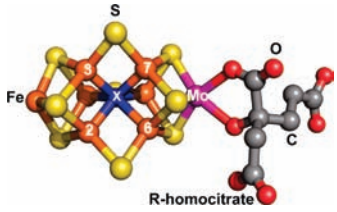


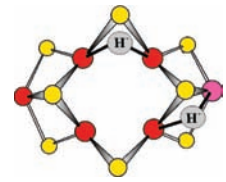
Figure 1. FeMo-co from PDB coordinate file 1M1N.pdb: Fe, rust; Mo, magneta; S, yellow; C, dark gray; O, red; X, blue.

Recent studies revealed the importance of substrate interactions at the 4Fe-4S face of FeMo-co defined by Fe atoms 2, 3, 6, and 7. Of particular importance, increasing the size of the side chain of residue α-70^{Val}, which lies over Fe6, by substitution with isoleucine leaves the reactivity of FeMo-co toward proton reduction unaltered but severely restricts the reduction of nitrogenous and alkyne substrates.[5,6] Although the changed substrate preferences of the α-70^{Ile} variant implicate Fe as the site of substrate binding, they do not preclude migration of substrate-derived moieties between metal atoms during reduction. As a result, we have initiated studies that directly probe the involvement of Mo during catalytic turnover.

The resting state wild type (WT) MoFe protein (E_0) shows a rhombic EPR spectrum from $S = 3/2$ FeMo-co that is conventionally treated in terms of a fictitious spin $S' = 1/2$ with g-tensor, $\mathbf{g}' = [4.32, 3.64, 2.0]$. When the α-$70^{Ile}$ MoFe protein is freeze-trapped during H^+ reduction under Ar, the majority of its E_0 EPR signal disappears and is replaced by the $S = 1/2$ signal ($g_{||} = 2.15$, $g_{2,3} = 2.01, 1.97$) of an intermediate that has been shown to be the pivotal E_4 MoFe state that is activated for N_2 binding and reduction through the accumulation of 4 electrons/protons by FeMo-co.[7,8] ENDOR studies of E_4 showed that it contains two hydrides bound to FeMo-co.[9] We here report a ^{95}Mo ENDOR study that determines whether Mo is involved in hydride binding.

The bound hydrides of E_4 have a large isotropic hyperfine coupling, $a_{iso} \approx 24$ MHz and an anisotropic contribution, $\mathbf{T} = \pm$ [−13.3, 0.7, 12.7] MHz, that exhibits almost complete rhombicity, defined by the form $\mathbf{T_{rh}} \approx \pm[t, 0, -t]$. This form rules out terminal hydrides, which would have a roughly axial $\mathbf{T}$[10] and suggests that the bound hydrides bridge two metal ions,[11] as Fe−H−Fe and/or Mo−H−Fe fragments (Chart 1), with Mo−H−Fe6 suggested by the indications that Fe6 participates in binding alkyne substrates.[5,6]

Chart 1. Representative Hydride Bridging Modes



Equations presented earlier for the anisotropic hyperfine interaction matrix, $\mathbf{T}$, of a nucleus that undergoes through-space dipolar interactions to the two metal ions of a spin-coupled dinuclear center[12] are straightforwardly generalized to describe an M_1-H-M_2 fragment in which M_1, M_2 are part of the multimetallic spin-coupled FeMo-co center. For a given M_1-H-M_2 geometry, the components of $\mathbf{T}$ become a function of the coefficients [K_1, K_2] that describe the projection of the total cluster spin on the local metal-ion spins. These equations are now applied to E_4.

Consider a hydride bound as a Mo−H−Fe fragment with the 2.7 Å Mo−Fe separation characteristic of FeMo-co and Fe−H and Mo−H bond lengths of ∼1.66 Å and ∼1.78 Å respectively, the most probable values for all Fe−H−M and Mo−H−M fragments in the Cambridge Structural Database. The observed $\mathbf{T}$ of E_4 can be matched only for $[K_{Fe}, K_{Mo}] \approx \pm[0.4-0.5, 0.4-0.3]$ and $\pm[\sim 0.3, -0.2]$, values that do not vary substantially with bond lengths. In short, Mo involvement in the hydride binding would require $|K_{Mo}| \gtrsim 0.2$. The K for an ion can be experimentally measured as the ratio of the isotropic hyperfine coupling, a_{iso}, for the spin-coupled metal ion to $a_{iso}{}^0$, that for the ion in the absence of spin coupling: $K = a_{iso}/a_{iso}{}^0$. We here determine K_{Mo} for the E_4 intermediate state through ENDOR measurements of the ^{95}Mo enriched MoFe protein, further comparing the results with those for the E_0 resting state.

The EPR spectrum of α-70^{Ile} MoFe in its E_0 state shows two $S = 3/2$ E_0 signals: one with $g_1(a)' = 4.36$ ($S' = 1/2$) corresponds to the signal for WT enzyme; the other has a slightly higher value, $g_1(b)' = 4.53$. These signals have been assigned to alternate conformations of amino acids near FeMo-co.[5,6]

Early ^{95}Mo CW X-band ENDOR measurements indicated that the resting-state WT MoFe protein contains a diamagnetic Mo(IV), an assignment based on its small hyperfine coupling, $a_{iso} \approx 6$ MHz (expressed in terms of the true, $S = 3/2$) and its quadrupolar interaction.[13] Figure 2, left, shows 35 GHz Davies pulsed ENDOR[14]

[†] Northwestern University.
[§] Utah State University.
[‡] Virginia Tech.

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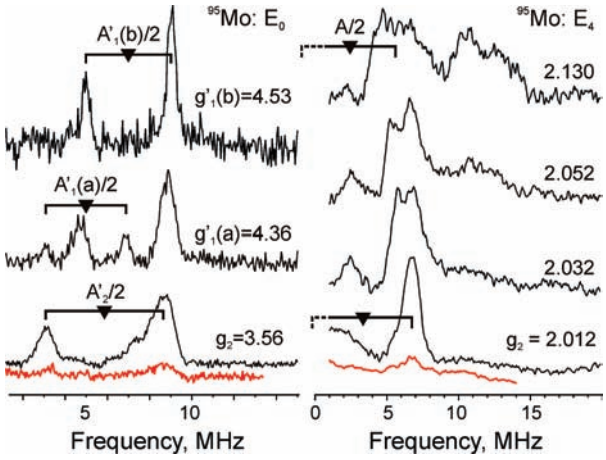

Figure 2. (Left) Davies ^{95}Mo-ENDOR spectra of ^{95}Mo-enriched (black) and natural-abundance (red) α-70^{Ile} MoFe protein in the resting state. (Right) CW ^{95}Mo-ENDOR spectra of ^{95}Mo-enriched (black) and natural-abundance (red) α-70^{Ile} MoFe protein in E_4 intermediate state. Brackets in left and right patterns indicate frequencies for $\nu_\pm$ doublets, as described in text. *Conditions*. $T = 2$ K. *Pulsed:* microwave frequency, ~34.78 GHz, Davies sequence, $\pi/2 = 40$ ns, $\tau = 600$ ns, RF 40 μs, random hop acquisition, 350−1500 scans. *CW:* microwave frequency, ~35.1 GHz; modulation amplitude, 4 G; time constant, 64 ms; RF sweep speed, 1 MHz/s; RF bandwidth-broadened to 100 kHz.

signals collected[15] at g_1' from the resting-state α-70^{Ile} MoFe protein with ^{95}Mo in natural abundance (15.8%) and isotopically enriched (~95% ^{95}Mo).[16] As the signals are ~6 times stronger for the enriched sample, they can be assigned, as before, to the $m_I = \pm 1/2$ transitions of ^{95}Mo ($I = 5/2$).

The ^{95}Mo spectrum from the α-70^{Ile} MoFe protein collected at g_1(b)′ = 4.53 shows a ν_+/ν_- doublet ($\nu_\pm = |\pm A'/2 + \nu_{Mo}|$) associated with the perturbed (b) conformation; it is centered at an effective hyperfine coupling, $A'(b)/2 = 7.01$ MHz, and split by $2\nu_{Mo}$, which is larger than the intrinsic value for the ^{95}Mo Larmor frequency, $2\nu_{Mo}{}^0$. The difference, ($\nu_{Mo}{}^0 - \nu_{Mo}$), is caused by the pseudonuclear Zeeman effect and can be used to calculate the zero-field splitting (Δ) between the ground $m_S = \pm 1/2$ and excited $m_S = \pm 3/2$ doublets of the $S = 3/2$ state:[17] Δ(b) = 9.6 cm^{-1}, notably less than Δ = 12.5 cm^{-1} previously reported for WT enzyme.[17]

When the field is increased to g_1(a)′ = 4.36, the (b) doublet persists with little change in coupling while a doublet from the (a) conformer appears (Figure 2). Its hyperfine coupling, $A'(a)/2 = 4.98$ MHz, and the zero-field splitting Δ(a) = 11.8 cm^{-1} are essentially the same as those for WT enzyme. Correction of the ^{95}Mo hyperfine couplings (A'; $S' = 1/2$) at g_1' to those for the true $S = 3/2$ spin ($A = (g_e/g_1')A'$) yields A(a) = 4.57 MHz, A(b) = 6.19 MHz. Spectra collected across the EPR spectrum of the WT MoFe protein show the hyperfine coupling to be roughly isotropic, with $a_{iso} \approx 6$ MHz ($S = 3/2$);[13] a similar set of spectra for the (a) conformation of the α-70^{Ile} variant yields an equivalent value, while that for the (b) conformation is larger, ~7 MHz. Surprisingly, this increase is larger than that for the Δ*NifV* mutant,[13] which contains an FeMo-co with homocitrate replaced by citrate.[1] These measurements thus reveal that structural perturbations in the vicinity of Fe6 caused by the α-70$^{Val\rightarrow Ile}$ modification are sensed at Mo.

Figure 2, right, shows 35 GHz CW ENDOR spectra[18] of ^{95}Mo-enriched intermediate E_4 obtained at several g-values. The signals seen with the enriched sample again can be assigned to ^{95}Mo from their absence in natural-abundance spectra. The ^{95}Mo spectrum obtained at $g_2 = 2.012$ shows a sharp ν_+ feature associated with an $m_I = \pm 1/2$, doublet centered $A/2 = 3$ MHz (true $S = 1/2$) and split by $2\nu_{Mo}{}^0$; the ν_- feature falls at ~0 MHz and is not detected. As the g-value of observation is increased, the ν_+ feature moves to lower frequency; it first splits and then broadens, with the coupling decreasing to $A/2 \approx 1.5$ MHz by $g \approx g_{||}$. In addition, satellite transitions associated with $m_I = \pm 3/2, 5/2$ transitions appear and increase in frequency. Regardless of whether the observed splitting arises from hyperfine anisotropy or from the presence of two conformers, simulations show that the isotropic ^{95}Mo coupling is $a_{iso} \approx 4$ MHz, *less* than that for the resting state.

The decrease in a_{iso} for ^{95}Mo of E_4 from the already small value in the resting state MoFe protein strongly suggests that the resting Mo(IV) is not one-electron reduced during the accumulation of the four electrons of E_4. In any case, the effective K for Mo is very small; a value of $a_{iso}{}^0 \gtrsim 100$ MHz is indicated by studies of mononuclear Mo complexes[19,20] and yields for E_4 an effective spin-coupling coefficient no greater than $|K_{Mo}| = |a_{iso}/a_{iso}{}^0| \lesssim 0.04$, at least 5-fold less than the lower bound, $|K_{Mo}| \gtrsim 0.2$, required for Mo to be involved in forming a Mo−H−Fe, hydride. As the hydride couplings also are both far too large, given the value of $|K_{Mo}|$, and of the wrong symmetry to be associated with a terminal hydride on Mo,[10] we may thus conclude that Mo does *not* participate in binding a hydride of the catalytically central E_4 intermediate and that only Fe ions are involved. Nonetheless, the response of the Mo coupling to subtle conformational changes in E_0 and to the formation of E_4 suggest that Mo is intimately involved in tuning the geometric and electronic properties of FeMo-co in these states. Similar investigations of Mo involvement in other intermediates are planned.

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