Supporting Information: Resolving the structure of the $E_1$ state of Mo Nitrogenase using Mo and Fe K-edge EXAFS and QM/MM calculations

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S1. EPR of the resting and low-flux turnover states of MoFe

EPR measurements were performed to establish the relative degree of reduction of the S = 3/2 signal of the E₀ resting state of MoFe N₂ase in turnover samples. These measurements were performed on the exact samples which were used for further XAS measurements. All continuous wave (CW) X-band EPR measurements were recorded using a Bruker E500 ELEXSYS spectrometer equipped with a Bruker dual-mode cavity (ER4116DM) and an Oxford Instruments helium flow cryostat (ESR 900). All measurements were performed at 10 K. A high-sensitivity Bruker Super-X (ER-049X) bridge with integrated microwave frequency counter was employed as the microwave unit. A magnetic field controller (ER032T) was externally calibrated with a Bruker NMR field probe (ER035M). X-band measurements utilized a 2 mW microwave power and 0.746 mT/100 kHz modulation. Spectra were analyzed in both the local software package esim_gfit (available from Eckhard Bill) and the software package EasySpin (version 5.1.9) as implemented in Matlab.¹ An average of 50% reduction in the S = 3/2 E₀ signal was found for turnover samples, based upon both relative intensity of the g₁ feature at g = 4.3 and spin-integration area (Figure S1-1).

Figure S1-1. X-band EPR spectra of the resting (E₀, black) and turnover (E₀+E₁, red) states. The large radical signal ~ g = 2 arises from the sample holder itself and is unaffiliated with the MoFe sample.
S2. Acquiring and adjusting degeneracy „$N$“

In EXAFS fitting, $N$ represents the degeneracy of a given scattering path. This can be interpreted as the average „coordination number“, or how many of a given scatterer surround the absorbing atom. In a more technical sense, it is the number of identical distinct ways per absorbing atom that the scattering defined by a given path can occur.

The degeneracy „$N$“ can be easily ascertained when crystal structures are available, or sufficient information about the coordination environment of the absorber of interest is already known. If we consider the case of the Mo EXAFS of MoFe, where Mo is the absorber, it is already established that Mo is coordinated by two O, one N, three S, and additionally has three neighboring Fe. If we fit individual scattering paths for each of these neighbors, we have $N_{Mo-O} = 2$, $N_{Mo-N} = 1$, $N_{Mo-S} = 3$, and $N_{Mo-Fe} = 3$. As O and N are virtually indistinguishable due to their similar mass, the ability to differentiate these paths depends on how disparate the Mo-O vs. Mo-N distances are. If we consider Mo-O and Mo-N indistinguishable, then simply $N_{Mo-O/N} = 3$, $N_{Mo-S} = 3$, and $N_{Mo-Fe} = 3$.

If we consider the case of Fe in MoFe, things become more complicated as there are 15 unique Fe in the protein which may absorb; while a possible partially occupied 16$^{th}$ mononuclear Fe site has been reported, it is not considered due to the uncertain occupancy of this site, light-atom only coordination, and small contribution to the total spectrum. To obtain $N$ here, we can begin by counting all of the unique scattering paths for each individual Fe. The unique scattering pathways for Fe are Fe-C, Fe-Mo, Fe-S, and Fe-Fe. We can then look at the distribution of these distances to decide whether they can be fit with one single-scattering path, or if multiple are needed. For example, a quick survey of a histogram of Fe-Fe distances (Figure S2-1) shows that there is a major group of short Fe-Fe distances between 2.5 and 3 Å, a second group of longer distances ~ 3.8 Å, and several even longer scatterers. Considering the resolution of EXAFS experiments are typically ~ 0.1-0.2 Å (determined by the equation $\Delta R = 2\pi/\Delta k$, where $\Delta R$ is the minimum difference in scattering distance to differentiate scatterers of the same identity, and $\Delta k$ is the range of k-space utilized in the fitting procedure), we can immediately split the Fe-Fe scattering shell into „short“ and „long“ Fe-Fe scattering paths.
We can further examine whether these Fe-Fe paths need to be further differentiated. Looking to Figure S2-2, we see that the long Fe-Fe scatterers arising from the P-cluster are considerably spread over a ~1.7 Å range of distances. To see whether some of these may be feasible to fit, we can determine $N$ for a hypothetical path by dividing the total number of scatterers described by that path by the total number of unique Fe (15). For example, a scattering path intended to describe the group of 6 near degenerate Fe-Fe scatterers ~4.6 Å would have $N = 0.4$. Considering the large disparity (and therefore low degeneracy) of the Fe-Fe(long) distances arising from the P-cluster, combined with their overall relatively long distance away from the absorber and the limited number of degrees of freedom available in our fitting procedure (determined by the Nyquist criterion, such that the number of degrees of freedom $X_{ind}$ is defined by $X_{ind} = \frac{2(k_{max}-k_{min})(R_{max}-R_{min})}{\pi}$), these scatterers should be omitted in a good EXAFS model of this system. Meanwhile, a scattering path intended to fit the 12 tightly grouped long Fe-Fe distances arising from FeMoco, which are spread by only ~0.04 Å, (inset of Figure S2, top) would have $N = 0.8$, and considering the reasonable distance and substantial degeneracy should be able to be reasonably modeled.

**Figure S2-1.** Histogram showing all of the Fe-Fe distances below 5.6 Å based on the XRD structure, PDB ID 3U7Q.

![Histogram showing all of the Fe-Fe distances below 5.6 Å based on the XRD structure, PDB ID 3U7Q.](image-url)
Figure S2-2. Enlarged version of Figure 8 from the main text. Comparison of the (top) long and (bottom) short Fe-Fe bond distances of P-cluster and (insets) FeMoco based on the XRD structure, PDB ID 3U7Q. For the P-cluster distances, both the broader histogram (light red, broad) and exact distances (red, narrow) have been provided. It should be immediately noticeable that the FeMoco presents a significantly more symmetric structure, with long Fe-Fe distances in FeMoco varying only $\Delta_{\text{Fe-Fe(long)}} \sim 0.04$ Å vs. $\Delta_{\text{Fe-Fe(long)}} \sim 0.75$-1.7 Å in the P-cluster, and short Fe-Fe distances only $\Delta_{\text{Fe-Fe(short)}} \sim 0.1$ Å vs. $\Delta_{\text{Fe-Fe(short)}} \sim 0.4$ Å.

Moving to the short Fe-Fe distances, we have a similar albeit less extreme situation. A scattering path intended to fit all short Fe-Fe distances would need to
encompass a total of 52 Fe-Fe scatterers (24 from FeMoco, 28 from the P-cluster), and would therefore have a degeneracy of $N \sim 3.47$. The 24 FeMoco short Fe-Fe scatterers are spread by $\sim 0.1 \text{ Å}$ with a median distance of $\sim 2.62 \text{ Å}$. Meanwhile, we see that the P-cluster short Fe-Fe distances are spread over a range of $\sim 0.4 \text{ Å}$, with 14 scatterers shorter than 2.7 Å, and 14 longer. Considering this relatively wide range of distances (and that the resolution of our fitting procedure ($\Delta R$) is at most 0.143 Å) it is reasonable that we consider splitting the short Fe-Fe scattering path into two separate paths. Exactly where the split is made is a matter of judgement, and influences the final degeneracy $N$ of each of the new paths. In the present study, we have opted to split at approximately the median of the short Fe-Fe scatterers, 2.7 Å, resulting in a shorter scattering path Fe-Fe (short 1) with 40 scatterers ($N \sim 2.53$) and a slightly longer scattering path Fe-Fe (short 2) with 14 scatterers ($N \sim 0.93$).
S3. The feasibility of fitting the Fe-C scatterer

It is now well established that the FeMoco cluster contains a light atom at its core which has been identified as a carbide.\textsuperscript{3-6} The knowledge of its existence naturally has implications for any structural modelling of the FeMoco. However, considering C is a very light atom (and thus a weak scatterer) which is coordinated to only 6 of 15 unique irons (giving a degeneracy of $N = 0.4$), is inclusion of the Fe-C scattering path really required?

![Figure S3-1](image)

**Figure S3-1.** $k^3$-weighted Fe K-edge EXAFS of the resting E\textsubscript{0} state and the 6-component fit to the data (top). Individual scattering path contributions to the total fit in the 6-component model.

Figure S3-1 demonstrates the contributions of each individual scattering path to the 6-component model fit, where the Fe-C scattering path clearly makes only a small contribution to the overall fit. While this may seem insignificant, it is still unclear exactly how modulation of the other paths to make-up for this small contribution in the absence of a Fe-C scattering path would influence the final fit distances. In Figure S3-2, the influence of the presence of the Fe-C scattering path on the final determined distances for the 6-component and 5-component models is shown. Namely, this figure displays the differences in the fit distances for the all other scattering pathways when the Fe-C scattering path is not included. While some very slight modulation occurs, all of these changes are within the error of the fit.
Figure S3-2. Differences in fit distances of the Fe K-edge EXAFS between models including/excluding the Fe-C scattering path. Fe-Fe denotes the Fe-Fe(short) paths, with Fe-Fe(short 1) to the left, and Fe-Fe(short 2) to the right.

The exact parameters for each scattering path, as well as final goodness of fit parameters for the inclusion/exclusion of the Fe-C scatterer, are provided in Table S5-2. On the basis of these statistics, as well as the stark demonstrations provided in Figures S3-1 and S3-2, it is clear that fitting of the Fe-C scattering path in the Fe K-edge EXAFS has a trivial influence on the overall fit. We conclude by acknowledging that its existence sets an a priori precedence for including this scattering path when modelling the Fe EXAFS of MoFe, but that its small magnitude and very, very minor influence on the overall fit imply any conclusions based on the fit parameters of this scattering path should be avoided.
S4. Why is $E_0$ important?

The parameter $E_0$ (not be confused with the enzyme resting state $E_0$) is often referred to as the edge energy, reference energy, or even as the inner potential of the absorber of interest. In a formal sense, this parameter functions as the energy which can eject a photoelectron with zero final momentum from a given electronic shell (in the case of K-edge XAS, the 1s). Since numerous electronic processes can occur at the K-edge, including shake-up and shake-down transitions, this parameter becomes difficult to precisely determine. Therefore, it is usually necessary to fit $E_0$ when modeling EXAFS. $E_0$ functions to define at what point the EXAFS region begins, as can be seen from equation 1:

$$k(E) = \frac{1}{\hbar} \sqrt{2m_e(E - E_0)}$$  \hspace{1cm} (1)

where $\hbar$ is the reduced Planck’s constant, $E$ is a given incident energy, and $m_e$ is the mass of an electron. Since $E_0$ determines the beginning of the EXAFS region, it should also be intuitive that the value of $E_0$ lies in neither the pre-edge region or EXAFS regions; instead, it should correspond to some point along the edge, and possibly up to the white-line region.

In the case of a single absorbing atom, there can only be one $E_0$ for all scattering paths. This is because $E_0$ is defined as a characteristic of the absorber itself, and is independent of the scattering path. If a different $E_0$ is provided for each scattering path, a change in phase of these paths relative to one another will occur. While doing this may provide fits that appear statistically reasonable, the determined distances are unphysical and can carry major errors. This is demonstrated in Figure S4-1/Table S4-1 for the MoCl$_3$ttcn complex, which consists of Mo coordinated by three S and three Cl. Here, we can see that while using drastically different values of $E_0$ for each path provides slightly better statistics in the fit, it results in the exchange of the fit Mo-Cl and Mo-S distances, which is chemically unreasonable.

Some samples may contain multiple metals of the same identity (such as Fe in MoFe in this study). If several absorbers contribute to the same spectrum but exist in drastically different chemical environments or oxidation states (for example, 4-sulfur coordinated Fe(II) vs. an Fe(IV)-oxo), it may be possible to model each site individually within a single set of data, if the data is of sufficiently high quality. Using the hypothetical example, it would then be prudent to use one value of $E_0$ for scattering
pathways involving one of the iron sites, and another $E_0$ for those involving the other. However, these values should still remain within a reasonable range of one another, on the order of the change in the edge position (usually with 1-4 eV).

In the case of MoFe, only one unique Mo center exists, and it is known that all Fe centers exist in either the Fe(II) or Fe(III) oxidation state, all predominately ligated by either cysteine or sulfide ligation. Therefore, only a single $E_0$ should be applied to all scattering paths when fitting either the Mo or Fe K-edge EXAFS. Furthermore, as no shift is observed in the position of the edge upon reduction to $E_1$, both the $E_0$ and $E_1$ states should have the same (or very similar) $E_0$.

![Figure S4-1](image)

**Figure S4-1.** Mo K-edge FT EXAFS of MoCl₃ttcn, comparing the influence of using separate values of $E_0$ for each path (right), versus using an identical $E_0$ for each path (left).

**Table S4-1.** Comparison of the influence of using separate values of $E_0$ for each path, versus using an identical $E_0$ for each path in the MoCl₃ttcn complex when fitting the Mo K-edge EXAFS. All fits were performed using a k-range of 3.0-12.5 Å⁻¹ and an R-range of 1.0-2.5 Å, along with a fixed $S_0^2 = 1.05$.

| Model | Path | N | $E_0$ (eV) | $\sigma^2$ ($10^{-3}$ Å²) | R (Å) | R-factor |
|-------|------|---|-----------|-----------------|------|----------|
| 3Cl/3S | Cl   | 3 | 20011.33  | 1.00            | 2.40 | 0.0032   |
|       | S    | 3 | 20002.96  | 1.69            | 2.40 | 0.0026   |
| 3Cl/3S | Cl   | 3 | 20018.99  | 3.87            | 2.52 | 0.0026   |
|       | S    | 3 | 20002.96  | 1.69            | 2.40 | 0.0026   |
Figure S5-1. (top) Mo K-edge XAS spectra of resting (E₀, black), turnover (E₀+E₁, red) and pure E₁ (blue) MoFe. (bottom) Differential N2ase Mo XAS spectra of E₁ – E₀ (blue) and (E₀+E₁) - E₀ (red). Standard deviations for the difference spectra are plotted.
Figure S5-2. (top) Fe K-edge XAS spectra of resting ($E_0$, black), turnover ($E_0+E_1$, red) and pure $E_1$ (blue) Mo Fe. (bottom) Differential N2ase Mo XAS spectra of $E_1 - E_0$ (blue) and ($E_0+E_1$) - $E_0$ (red), demonstrating the changes which occur in these spectra upon native reduction. Standard deviations for both XAS and difference spectra are displayed. The spectra exhibit a pre-edge feature at 7112.8 eV and an inflection in the rising edge at 7118.4 eV. The pre-edge feature arises from the $1s \rightarrow 3d$ transition, which, while formally dipole forbidden, gains intensity through $T_d$-symmetry allowed $3d$-$4p$ mixing. Upon reduction, decreases in intensity at the pre-edge and rising edge are followed by an increased white-line intensity.
Figure S5-3. Comparison the |FT| space fits of the Mo K-edge EXAFS for the (left) 3-component and (right) 4-component models. The red dashed line represents the total fit, including all scattering paths. The spectra have been phase shifted for the Mo-S scattering path.

Figure S5-4. Comparison the |FT| space fits and Fe-S and Fe-Fe (short) scattering paths of the Fe K-edge EXAFS for the 5-component, $N_{\text{Fe-Fe(short)}} = 3.46$ model. The red dashed line represents the total fit, including all scattering paths (and not only those additionally displayed). The spectrum has been phase shifted for the Fe-S scattering path.
Figure S5-5. Comparison the |FT| space fits and Fe-S and Fe-Fe(short) scattering paths of the Fe K-edge EXAFS for the 5-component, $N_{\text{Fe-Fe(short)}} = 1.65$ model. The red dashed line represents the total fit, including all scattering paths (and not only those additionally displayed). The spectrum has been phase shifted for the Fe-S scattering path.

Figure S5-6. Comparison the |FT| space fits and Fe-S, Fe-Fe (short 1), and Fe-Fe (short 2) scattering paths of the Fe K-edge EXAFS for the 6-component, $N_{\text{Fe-Fe(short 1)}} = 2.53$ & $N_{\text{Fe-Fe(short 2)}} = 0.93$ model. The red dashed line represents the total fit, including all scattering paths (and not only those additionally displayed). The spectrum has been phase shifted for the Fe-S scattering path.
Figure S5-7. Comparison the |FT| space Fe-S, Fe-Fe(short 1), and Fe-Fe(short 2) scattering paths of the Fe K-edge EXAFS for the 6-component model as applied to the turnover $E_0+E_1$ state. The red dashed line represents the total fit, including all scattering paths (and not only those additionally displayed). The spectra have been phase shifted for the Fe-S scattering path.

Figure S5-8. Comparison the |FT| space Fe-S, Fe-Fe(short 1), and Fe-Fe(short 2) scattering paths of the Fe K-edge EXAFS for the 6-component model as applied to the $E_1$ state. The red dashed line represents the total fit, including all scattering paths (and not only those additionally displayed). The spectra have been phase shifted for the Fe-S scattering path.
Figure S5-9. Comparison of variation in determined bond distances from the resting $E_0$ state in the a) 4-component (reduced Fe-Fe(short) $N$, no C), b) 5-component (reduced Fe-Fe(short) $N$), c) 5-component (split Fe-Fe(short), no C), and d) 6-component models applied to the Fe EXAFS. Distances are calculated by subtraction of the fit $E_0$ distances from those of the turnover ($E_0+E_1$) and $E_1$ fits. The differences in the Fe-Fe(short) distances in c) and d) are split such that Fe-Fe(short 1) is to the left, and Fe-Fe(short 2) to the right.
### Table S5-1. Comparison of the fit parameters of the 3-component and 4-component Mo K-edge EXAFS models.

**Mo feff fits, $E_o = 20013.895$ eV**

| Sample | Path = 3 | $N$ | $S_0^2$ | $\sigma^2 (10^{-3} \text{ Å}^2)$ | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|--------|----------|-----|---------|----------------|-------|-------------|---------|
| Resting | O/N | 3 | 4.20 (1.01) | 2.217 (0.009) | 2 – 16.5 | 0.0061 |
| | S | 3 | 2.42 (0.30) | 2.362 (0.003) | min. $\Delta R$ |
| | Fe-short | 3 | 3.25 (0.18) | 2.689 (0.002) | 0.108 |
| Turnover | O/N | 3 | 3.52 (1.01) | 2.221 (0.009) | 2 – 16.5 | 0.0065 |
| $(E_0 + E_1)$ | S | 3 | 2.57 (0.36) | 2.361 (0.004) | min. $\Delta R$ |
| | Fe-short | 3 | 3.24 (0.19) | 2.688 (0.002) | 0.108 |
| Turnover | O/N | 3 | 2.34 (1.31) | 2.221 (0.010) | 2 – 16 | 0.0117 |
| $(E_1)$ | S | 3 | 2.81 (0.68) | 2.365 (0.005) | min. $\Delta R$ |
| | Fe-short | 3 | 3.16 (0.29) | 2.697 (0.003) | 0.112 |

| Sample | Path = 4 | $N$ | $S_0^2$ | $\sigma^2 (10^{-3} \text{ Å}^2)$ | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|--------|----------|-----|---------|----------------|-------|-------------|---------|
| Resting | O | 2 | 2.36 (0.67) | 2.198 (0.007) | 1.6 – 16.4 min. $\Delta R$ 0.106 | 0.0024 |
| | N | 1 | 1.72 (1.09) | 2.303 (0.011) | |
| | S | 3 | 2.51 (0.20) | 2.365 (0.004) | |
| | Fe-short | 3 | 3.18 (0.11) | 2.693 (0.001) | |
| Turnover | O | 2 | 2.53 (0.68) | 2.197 (0.006) | 1.6 – 16.4 min. $\Delta R$ 0.106 | 0.0024 |
| $(E_0 + E_1)$ | N | 1 | 2.01 (1.35) | 2.309 (0.011) | |
| | S | 3 | 2.24 (0.16) | 2.361 (0.002) | |
| | Fe-short | 3 | 3.16 (0.11) | 2.688 (0.001) | |
| Turnover | O | 2 | 0.93 (0.77) | 2.202 (0.011) | 1.6 – 16 min. $\Delta R$ 0.109 | 0.0108 |
| $(E_1)$ | N | 1 | 3.00 (0.02) | 2.302 (0.006) | |
| | S | 3 | 2.57 (0.52) | 2.367 (0.006) | |
| | Fe-short | 3 | 3.18 (0.26) | 2.697 (0.003) | |

### Table S5-2. Fe K-edge EXAFS fit parameters of the 4-component model using a reduced degeneracy $N$ for the short Fe-Fe path and no Fe-C scattering path (no Fe-C analog of 5-component model w/ reduced N).

**Fe feff fits, $E_o = 7118.03$ eV**

| Sample | Path = 4 | $N$ | $S_0^2$ | $\sigma^2 (10^{-3} \text{ Å}^2)$ | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|--------|----------|-----|---------|----------------|-------|-------------|---------|
| Resting | Mo | 0.2 | 2.00 (2.07) | 2.700 (0.026) | 2 – 15.8 min. $\Delta R$ 0.114 | 0.0252 |
| | S | 3.6 | 5.66 (0.39) | 2.269 (0.003) | |
| | Fe-short | 1.65 | 4.26 (0.96) | 2.612 (0.006) | |
| | Fe-long | 0.8 | 2.00 (1.08) | 3.691 (0.012) | |
| Turnover | Mo | 0.2 | 1.14 (1.16) | 2.704 (0.015) | 2 – 15.8 min. $\Delta R$ 0.114 | 0.0203 |
| $(E_0 + E_1)$ | S | 3.6 | 6.30 (0.42) | 2.277 (0.003) | |
| | Fe-short | 1.65 | 5.56 (0.94) | 2.612 (0.007) | |
| | Fe-long | 0.8 | 2.01 (1.00) | 3.704 (0.011) | |
| Turnover | Mo | 0.2 | 7.00 (0.00) | 2.664 (0.007) | 2 – 13 min. $\Delta R$ 0.143 | 0.0099 |
| $(E_1)$ | S | 3.6 | 7.28 (0.42) | 2.283 (0.003) | |
| | Fe-short | 1.65 | 5.48 (0.49) | 2.588 (0.004) | |
| | Fe-long | 0.8 | 4.37 (1.68) | 3.715 (0.016) | |
Table S5-3. Fe K-edge EXAFS fit parameters of the 5-component model using a reduced degeneracy $N$ for the short Fe-Fe path.

| Sample   | Path = 5 | $N$ | $S_0^2$ | $\sigma^2$ (10$^{-3}$ \(\text{Å}^2\)) | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|----------|----------|-----|---------|----------------------------------|---------|--------------|----------|
| Resting  | C        | 0.4 | 2.00 (0.23) | 1.910 (0.036) | 2 – 15.8 | min. $\Delta R$ 0.0224 |
|          | Mo       | 0.2 | 2.00 (2.08) | 2.700 (0.026) |         |              |          |
|          | S        | 3.6 | 5.66 (0.39) | 2.269 (0.003) |         |              |          |
|          | Fe-short | 1.65 | 4.26 (0.97) | 2.611 (0.006) | 0.114  |              |          |
|          | Fe-long  | 0.8 | 2.00 (0.05) | 3.691 (0.012) |         |              |          |
| Turnover | C        | 0.4 | 5.00 (0.00) | 1.949 (0.065) | 2 – 15.8 | min. $\Delta R$ 0.0201 |
| (E$_0 + E_1$) | Mo    | 0.2 | 1.15 (1.26) | 2.703 (0.016) |         |              |          |
|          | S        | 3.6 | 6.19 (0.44) | 2.277 (0.004) |         |              |          |
|          | Fe-short | 1.65 | 5.51 (1.02) | 2.612 (0.007) | 0.114  |              |          |
|          | Fe-long  | 0.8 | 2.00 (1.08) | 3.705 (0.012) |         |              |          |
| Turnover | C        | 0.4 | 5.00 (0.00) | 2.009 (0.073) | 2 – 13  | min. $\Delta R$ 0.0130 |
| (E$_1$)  | Mo       | 0.2 | 7.00 (0.00) | 2.679 (0.123) |         |              |          |
|          | S        | 3.6 | 7.22 (0.59) | 2.276 (0.004) |         |              |          |
|          | Fe-short | 1.65 | 5.39 (1.48) | 2.591 (0.006) | 0.143  |              |          |
|          | Fe-long  | 0.8 | 4.87 (2.37) | 3.714 (0.022) |         |              |          |

Table S5-4. Fe K-edge EXAFS fit parameters of the 5-component model as applied to the resting state $E_0$ EXAFS, along with the 4-component analog in which the Fe-C scattering path is not considered.

| Sample   | Path = 4 | $N$ | $S_0^2$ | $\sigma^2$ (10$^{-3}$ \(\text{Å}^2\)) | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|----------|----------|-----|---------|----------------------------------|---------|--------------|----------|
| Resting  | Mo       | 0.2 | 1.00 (1.53) | 2.702 (0.017) | 2 – 15.8 | min. $\Delta R$ 0.0678 |
|          | S        | 3.6 | 5.90 (0.65) | 2.265 (0.006) |         |              |          |
|          | Fe-short | 3.64 | 8.00 (0.01) | 2.618 (0.008) | 0.114  |              |          |
|          | Fe-long  | 0.8 | 0.87 (1.25) | 3.690 (0.015) |         |              |          |

| Sample   | Path = 5 | $N$ | $S_0^2$ | $\sigma^2$ (10$^{-3}$ \(\text{Å}^2\)) | $R$ (Å) | $k$ (Å$^{-1}$) | R-factor |
|----------|----------|-----|---------|----------------------------------|---------|--------------|----------|
| Resting  | C        | 0.4 | 1.00 (4.84) | 1.907 (0.049) | 2 – 15.8 | min. $\Delta R$ 0.0684 |
|          | Mo       | 0.2 | 1.00 (1.68) | 2.702 (0.019) |         |              |          |
|          | S        | 3.6 | 5.90 (0.72) | 2.265 (0.006) |         |              |          |
|          | Fe-short | 3.64 | 8.00 (0.01) | 2.618 (0.008) | 0.114  |              |          |
|          | Fe-long  | 0.8 | 0.87 (1.34) | 3.690 (0.016) |         |              |          |
Table S5-5. Fe K-edge EXAFS fit parameters of the 5-component model using split short Fe-Fe paths and no Fe-C scattering path (a no Fe-C analog of 6-component model).

Fe feff fits, $E_0 = 7118.03$ eV

| Sample          | Path = 5 | $N$ | $S_0^2$ | $\sigma^2 \times 10^{-3}$ Å$^2$ | R (Å)   | $k$ (Å$^{-1}$) | R-factor |
|-----------------|----------|-----|---------|-------------------------------|---------|---------------|----------|
| Resting Mo      | 0.2      |     | 2.00 (0.26) | 2.683 (0.048)  | 2 – 15.8 |               |          |
|                 | S        | 3.6 | 5.63 (0.43) | 2.267 (0.004)  | 0.114    |               | 0.018    |
|                 | Fe-short | 2.53| 5.73 (1.27) | 2.622 (0.004)  | 0.114    |               | 0.018    |
|                 | Fe-short | 0.93|         | 2.855 (0.016)  |          |               |          |
|                 | Fe-long  | 0.8 | 2.00 (0.59) | 3.691 (0.013)  |          |               |          |
| Turnover Mo     | 0.2      |     | 2.00 (2.45) | 2.683 (0.035)  | 2 – 15.8 |               | 0.0153   |
| (E$_0 + E_1$)   | S        | 3.6 | 6.22 (0.44) | 2.274 (0.004)  | 0.114    |               | 0.0081   |
|                 | Fe-short | 2.53| 6.61 (1.27) | 2.620 (0.009)  | 0.114    |               | 0.0081   |
|                 | Fe-short | 0.93|         | 2.860 (0.027)  |          |               |          |
|                 | Fe-long  | 0.8 | 2.00 (0.98) | 3.704 (0.011)  |          |               |          |
| Turnover Mo     | 0.2      |     | 5.00 (0.00) | 2.664 (0.002)  | 2 – 13   |               | 0.143    |
| (E$_1$)         | S        | 3.6 | 7.54 (0.76) | 2.280 (0.005)  | 0.114    |               | 0.0081   |
|                 | Fe-short | 2.53| 7.47 (0.77) | 2.601 (0.005)  | 0.114    |               | 0.0081   |
|                 | Fe-short | 0.93|         | 2.849 (0.021)  |          |               |          |
|                 | Fe-long  | 0.8 | 5.05 (2.45) | 3.715 (0.022)  |          |               |          |

Table S5-6. Fe K-edge EXAFS fit parameters of the 6-component model. Due to the similar scattering behavior of the two Fe-short scattering paths, the $\sigma^2$ of these two paths were set to be equivalent during fitting.

Fe feff fits, $E_0 = 7118.03$ eV

| Sample          | Path = 6 | $N$ | $S_0^2$ | $\sigma^2 \times 10^{-3}$ Å$^2$ | R (Å)   | $k$ (Å$^{-1}$) | R-factor |
|-----------------|----------|-----|---------|-------------------------------|---------|---------------|----------|
| Resting C       | 0.4      |     | 2.00 (0.82) | 1.911 (0.034)  | 2 – 15.8 |               | 0.0142   |
|                 | Mo       | 2.0 | 2.00 (0.62) | 2.683 (0.013)  |          |               |          |
|                 | S        | 3.6 | 5.59 (0.44) | 2.267 (0.002)  |          |               |          |
|                 | Fe-short | 2.53| 5.72 (1.30) | 2.622 (0.003)  |          |               |          |
|                 | Fe-short | 0.93|         | 2.854 (0.012)  |          |               |          |
|                 | Fe-long  | 0.8 | 1.99 (0.09) | 3.691 (0.010)  |          |               |          |
| Turnover C      | 0.4      |     | 2.00 (2.81) | 1.928 (0.024)  | 2 – 15.8 |               | 0.0093   |
| (E$_0 + E_1$)   | Mo       | 2.0 | 2.00 (2.27) | 2.679 (0.010)  |          |               |          |
|                 | S        | 3.6 | 6.10 (0.39) | 2.273 (0.001)  |          |               |          |
|                 | Fe-short | 2.53| 6.45 (1.11) | 2.620 (0.003)  |          |               |          |
|                 | Fe-short | 0.93|         | 2.858 (0.010)  |          |               |          |
|                 | Fe-long  | 0.8 | 1.99 (0.85) | 3.704 (0.007)  |          |               |          |
| Turnover C      | 0.4      |     | 2.00 (0.35) | 1.992 (0.029)  | 2 – 13   |               | 0.0079   |
| (E$_1$)         | Mo       | 2.0 | 5.00 (0.00) | 2.664 (0.001)  |          |               |          |
|                 | S        | 3.6 | 7.12 (0.00) | 2.283 (0.003)  |          |               |          |
|                 | Fe-short | 2.53| 7.60 (0.91) | 2.603 (0.003)  |          |               |          |
|                 | Fe-short | 0.93|         | 2.856 (0.010)  |          |               |          |
|                 | Fe-long  | 0.8 | 4.66 (2.10) | 3.713 (0.014)  |          |               |          |
S6. Computational Details

S6.1. Labelling scheme of the broken-symmetry states & Selection of broken-symmetry states for investigation

The FeMoco cluster contains 8 metal centers with unpaired electrons. The $E_0$ state is calculated assuming the charge state $[\text{MoFe}_7\text{S}_9\text{C}]^{1-}$ (as has been established in previous work\textsuperscript{10,11}) that can be written in terms of a formal oxidation state distribution of $\text{Mo}^{\text{III}}4\text{Fe}^{\text{III}}3\text{Fe}^{\text{II}}$. The $\text{Mo}^{\text{III}}$ oxidation state has been previously determined by Mo HERFD-XAS and theoretical calculations.\textsuperscript{12} In $E_1$ the formal oxidation state distribution changes to $\text{Mo}^{\text{III}}3\text{Fe}^{\text{III}}4\text{Fe}^{\text{II}}$. To find the lowest energy antiferromagnetically coupled broken-symmetry spin states we first calculate high-spin states (where all spins are ferromagnetically aligned), $M_S = \frac{35}{2}$ ($E_0$) and $M_S = \frac{34}{2}$ ($E_1$). From these high-spin states, the spin densities at selected Fe atoms are “flipped” and the SCF (self-consistent field) is then reconverged to find the lowest energy solution corresponding to a specific $M_S$ value. For $E_0$, the $M_S = \frac{3}{2}$ solutions are lowest in energy, consistent with the experimental $S = \frac{3}{2}$ spin state. As the experimental spin state of $E_1$ is $\geq 1$,\textsuperscript{13} both the $M_S = 1$ and $M_S = 2$ broken symmetry state solutions were initially probed (Figure S6.1-1).

![Energy vs. Labeling Scheme](image)

**Figure S6.1-1.** Relative energies (w.r.t. BS7-235) of possible broken-symmetry solutions from the addition of a single electron to the BS-235 optimized $E_0$ structure of the FeMoco cluster to form an unrelaxed unprotonated $E_1$ state. The presented labeling scheme provides first the $C_{3v}$ category of the BS solution, followed by three numbers which describe the BS solution under $C_1$ symmetry. The numbers denote the “spin-down” Fe ions.
The possible BS solutions of the E\textsubscript{0} state of FeMoco have previously been organized into 10 categories with the assumption that the cluster maintains C\textsubscript{3v} symmetry\textsuperscript{14}. However, as the effective C\textsubscript{3v} symmetry of the cluster is broken by the ligands and the secondary environment of the protein, all degeneracy within these categories is lifted, resulting in up to 35 different solutions. The same case is true following the addition of one electron to form the E\textsubscript{1} state. The energies of these different solutions for E\textsubscript{1} (in the absence of an added proton) are shown in Figure S6.1-1. From these calculations, we find that all three of the lowest energy solutions fall within the “BS7” category, with S = 2. Any further discussion of these three lowest energy solutions will forgo the label BS7.

The precise couplings of the BS-235 solution of E\textsubscript{0} and three lowest energy BS solutions of E\textsubscript{1} (BS-235, BS-346 and BS-247) are described in Figure S6.1-2. Details regarding the electronic state of E\textsubscript{0} can be found in our past work\textsuperscript{10-12}. Briefly, based on localized orbital (Pipek-Mezey method\textsuperscript{15}) and structural analyses, our present understanding of E\textsubscript{0} consists of an Mo\textsuperscript{III}4Fe\textsuperscript{III}3Fe\textsuperscript{II} oxidation state distribution in a BS-235 electronic configuration. In this case, Fe2 and Fe3 form a mixed-valent delocalized pair in the [Fe\textsubscript{4}S\textsubscript{3}C] sub-cubane, while Fe6 and Fe7 are mixed-valence delocalized in the [MoFe\textsubscript{3}S\textsubscript{3}C] sub-cubane. There is partial delocalization of the paired electron between Fe1 and Fe4, although it is more localized on Fe1. The BS-247 and BS-346 solutions, which are comparable in energy to the BS-235 solution, are the C\textsubscript{3} rotated equivalents of this description.

In the context of E\textsubscript{1}, these three solutions effectively rotate which of two possible Fe in the cluster is reduced (Fe\textsubscript{4} or Fe\textsubscript{5} in BS-235, Fe\textsubscript{2} or Fe\textsubscript{6} in BS-346, and Fe\textsubscript{3} or Fe\textsubscript{7} in BS-247; see Figure S6.1-2). While it is possible that the added electron could be on either of two Fe atoms for a given BS solution, we have found that reduction is always localized to one of the Fe atoms of the [MoFe\textsubscript{3}S\textsubscript{3}C] sub-cubane (Fe\textsubscript{5} through Fe\textsubscript{7}). The localized orbital of the added electron are provided in Figure S6.1-2 for the most favorable BS solution of each of the three possible belt-sulfide protonation sites. Interestingly, localized orbital analysis of these solutions has revealed that the delocalized electron of the spin-down mixed-valent delocalized pair in the [Fe\textsubscript{4}S\textsubscript{3}C] sub-cubane becomes less delocalized in our E\textsubscript{1} models. While this localization tends to occur at the Fe of the pair which resides closer to either of the Arg residues (Arg 96 or Arg359, Figure S6.4-2), we note that the magnitude of localization/delocalization is dependent on both the size of the QM region and employed exchange-correlation
Tables S6.1-1 to S6.1-3 give the spin populations of all broken-symmetry states calculated for $E_0$ and $E_1$. The site of reduction can also be deduced from the change in spin population.

**Figure S6.1-2.** Spin-coupling of the BS235 solution of the $E_0$ state, and the three considered BS solutions (BS235, BS346, and BS247) resulting broken symmetry solutions of the $E_1$ state (demonstrated for the S2B(H)-BS346, S3A(H)-BS235, and S5A(H)-BS247 models) according to localized orbital analysis. The red arrow denotes completely spin-delocalized mixed-valent pairs. In all cases, the additional electron of the $E_1$ state (blue) most favorably resides on the a) Mo sub-cubane, and b) on the Fe neighboring the protonated S. The localized $d$-orbital associated with the minority-spin electron at Fe6, Fe5 and Fe7, respectively is shown.

**Table S6.1-1** Mulliken spin-populations of the metal centers of FeMoco for all homocitrate-protonated models of $E_0$ and $E_1$ using the BS-235 solution.

| Model       | Metal Center | $N_e$ | Mo   | Fe1  | Fe2   | Fe3   | Fe4   | Fe5   | Fe6   | Fe7   |
|-------------|--------------|-------|------|------|-------|-------|-------|-------|-------|-------|
| $E_0$       |              |       | -0.54| 3.43 | -3.21 | -3.21 | 3.30  | -3.12 | 2.98  | 2.90  |
| $E_0 + e^-$ |              |       | -0.45| 3.42 | -3.17 | -3.17 | 3.36  | -2.76 | 3.06  | 2.94  |
| S2B(H)      |              |       | -0.36| 3.41 | -3.14 | -3.21 | 3.37  | -2.77 | 2.99  | 3.00  |
| S5A(H)      |              |       | -0.59| 3.46 | -3.18 | -3.21 | 3.21  | -2.51 | 3.05  | 2.99  |
| S3A(H)      |              |       | -0.40| 3.41 | -3.18 | -3.14 | 3.35  | -2.74 | 3.08  | 2.90  |
| S1A(H)      |              |       | -0.43| 3.38 | -3.06 | -3.16 | 3.22  | -2.75 | 3.12  | 2.99  |
| S3B(H)      |              |       | 0.22 | 3.43 | -3.18 | -3.21 | 3.34  | -3.13 | 3.17  | 2.87  |
| Fe6(H)      |              |       | -0.36| 3.40 | -2.70 | -3.05 | 3.32  | -2.65 | 2.35  | 2.96  |

| Model       | Metal Center | $N_e$ | Mo   | Fe1  | Fe2   | Fe3   | Fe4   | Fe5   | Fe6   | Fe7   |
|-------------|--------------|-------|------|------|-------|-------|-------|-------|-------|-------|
| $E_1$       |              |       |      |      |       |       |       |       |       |       |

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### Table S6.1-2 Mulliken spin-populations of the metal centers of FeMoco for all homocitrate-protonated models of E₀ and E₁ using the BS-346 solution.

| Metal Center | Nₛ | Mo | Fe1 | Fe2 | Fe3 | Fe4 | Fe5 | Fe6 | Fe7 |
|--------------|----|----|-----|-----|-----|-----|-----|-----|-----|
| E₀           |    | -0.26 | 3.42 | -3.23 | -3.20 | 2.99 | -3.12 | 2.87 |
| E₀ + e⁻      |    | -0.31 | 3.46 | -3.21 | -3.19 | 3.06 | -2.62 | 2.94 |
| S2B(H)       |    | -0.23 | 3.45 | -3.14 | -2.78 | 2.90 | -3.09 | 2.92 |
| S5A(H)       |    | -0.24 | 3.41 | -3.12 | -3.20 | 3.09 | -2.69 | 2.86 |
| S3A(H)       |    | -0.28 | 3.42 | -3.16 | -3.22 | 3.10 | -2.71 | 2.95 |
| S1A(H)       |    | -0.08 | 3.43 | -3.20 | -3.22 | 3.00 | -2.83 | 2.95 |
| S3B(H)       |    | -0.35 | 3.43 | -3.12 | -3.13 | 3.04 | -2.61 | 2.87 |

### Table S6.1-3 Mulliken spin-populations of the metal centers of FeMoco for all homocitrate-protonated models of E₀ and E₁ using the BS-247 solution.

| Metal Center | Nₛ | Mo | Fe1 | Fe2 | Fe3 | Fe4 | Fe5 | Fe6 | Fe7 |
|--------------|----|----|-----|-----|-----|-----|-----|-----|-----|
| E₀           |    | -0.46 | 3.42 | -3.21 | 3.32 | -3.18 | 3.00 | 2.93 | -3.05 |
| E₀ + e⁻      |    | -0.36 | 3.39 | -3.16 | 3.38 | -3.19 | 3.09 | 2.95 | -2.62 |
| S2B(H)       |    | -0.32 | 3.40 | -3.19 | 3.40 | -3.07 | 2.90 | 3.03 | -2.65 |
| S5A(H)       |    | -0.46 | 3.44 | -3.18 | 3.28 | -3.18 | 3.07 | 2.99 | -2.55 |
| S3A(H)       |    | -0.38 | 3.42 | -3.08 | 3.39 | -3.15 | 3.05 | 2.98 | -2.66 |
| S1A(H)       |    | -0.26 | 3.42 | -3.11 | 3.36 | -3.15 | 2.99 | 2.98 | -2.78 |
| S3B(H)       |    | -0.36 | 3.38 | -2.76 | 3.35 | -3.06 | 3.04 | 2.33 | -2.47 |

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The protonation state of the hydroxyl group of homocitrate has direct implications on the structural environment of Mo in the FeMoco cluster. Previous studies have supported that this group is protonated in the E₀ state, and is held hydrogen-bonded to one of the uncoordinated carboxylate groups of the homocitrate (Figure S6.2-1). However, it is unknown whether this remains true for any of the Eₙ states (n > 0), and necessitates further investigation in the case of E₁.

To investigate the possibility of the hydroxyl group homocitrate undergoing deprotonation to form E₁, we have chosen to investigate several BS solutions of the two most energetically favorable protonation sites (S2B and S5A). Comparing the structural changes which occur at Mo in going from E₀ to a hydroxyl-deprotonated E₁ state (Figure S6.2-2), we find that several significant changes occur. In four of the five cases, a drastic contraction in the Mo-O distance; only the S5A(H) model using a BS247 solution appears reasonable. However, this same solution also displays a drastic increase in the average Mo-S distance, and contraction in the average Mo-Fe distance. The four other solutions show expansion of both the average Mo-S and Mo-Fe distances, well out of the range of experimental error.
Figure S6.2. Comparison of the changes in Mo bond distances for several protonated models of E₁ in which the homocitrate hydroxyl group is unprotonated. Experimental distances and standard errors are provided as solid lines and corresponding shaded areas spanning the width of each graph.

Meanwhile, we find that deprotonation of the hydroxyl group during E₁ formation has very little effect on the average Fe-S, Fe-Fe(short), and Fe-Fe(long) distances (Figure S6.2-3), where the changes observed are similar to those found when the hydroxyl group is protonated. This is not so surprising, as the question of homocitrate protonation state predominately concerns the Mo coordination environment, and only several of the Fe secondarily.

The drastic changes predicted to occur in the Mo coordination environment during formation of E₁ in the absence of a hydroxyl proton are unsupportive of this site being deprotonated during reduction. Furthermore, we find that all E₁ models investigated here, which lack a protonated hydroxyl group, produce a series of HOMOs with positive orbital energies (Tables S6.2-1 through S6.2-5). This is an unphysical result, as it implies the occupation of an orbital which is so destabilized that spontaneous ionization would occur. In all cases, only the addition of a H⁺ back to the model produces negative energies for all HOMOs.
Figure S6.2-3. Comparison of the changes in Fe bond distances for several protonated models of $E_1$ in which the homocitrate hydroxyl group is unprotonated. Experimental distances and standard errors are provided as solid lines and corresponding shaded areas spanning the width of each graph.

Table S6.2-1. Calculated alpha and beta frontier orbital energies of the unprotonated homocitrate S2B(H) model of $E_1$, calculated using the BS-235 solution.

| Orbital | $\alpha$ (eV) | Orbital | $\beta$ (eV) |
|---------|---------------|---------|--------------|
| LUMO    | 2.9501        | LUMO    | 2.9291       |
| HOMO    | 1.7054        | HOMO    | 1.1761       |
| HOMO-1  | 1.1893        | HOMO-1  | 1.1051       |
| HOMO-2  | 0.9646        | HOMO-2  | 1.0225       |
| HOMO-3  | 0.8322        | HOMO-3  | 0.8506       |
| HOMO-4  | 0.7821        | HOMO-4  | 0.4976       |
| HOMO-5  | 0.559         | HOMO-5  | 0.3563       |
| HOMO-6  | 0.3763        | HOMO-6  | 0.2054       |
| HOMO-7  | 0.1549        | HOMO-7  | 0.1078       |
| HOMO-8  | -0.0394       | HOMO-8  | 0.0105       |

Table S6.2-2. Calculated alpha and beta frontier orbital energies of the unprotonated homocitrate S5A(H) model of $E_1$, calculated using the BS-235 solution.

| Orbital | $\alpha$ (eV) | Orbital | $\beta$ (eV) |
|---------|---------------|---------|--------------|
| LUMO    | 2.9455        | LUMO    | 2.8856       |
| HOMO    | 1.5229        | HOMO    | 1.1629       |
| HOMO-1  | 1.2197        | HOMO-1  | 1.1091       |
| HOMO-2  | 0.9956        | HOMO-2  | 1.0122       |
| HOMO-3  | 0.8268        | HOMO-3  | 0.783        |
| HOMO-4  | 0.7857        | HOMO-4  | 0.5739       |
| HOMO-5  | 0.4991        | HOMO-5  | 0.3986       |
| HOMO-6  | 0.4591        | HOMO-6  | 0.2641       |
| Orbital | Energy (eV)    | Orbital | Energy (eV)    |
|---------|---------------|---------|---------------|
| HOMO-7  | 0.2027        | HOMO-7  | 0.0965        |
| HOMO-8  | 0.0428        | HOMO-8  | 0.0424        |
| HOMO-9  | -0.0853       | HOMO-9  | 0.0319        |

Table S6.2-3. Calculated alpha and beta frontier orbital energies of the unprotonated homocitrate S2B(H) model of E1, calculated using the BS-346 solution.

| Orbital | Energy (eV)    | Orbital | Energy (eV)    |
|---------|---------------|---------|---------------|
| LUMO    | 2.9803        | LUMO    | 2.872         |
| HOMO    | 1.2957        | HOMO    | 1.2335        |
| HOMO-1  | 1.1226        | HOMO-1  | 1.1134        |
| HOMO-2  | 0.887         | HOMO-2  | 1.086         |
| HOMO-3  | 0.863         | HOMO-3  | 0.8018        |
| HOMO-4  | 0.6499        | HOMO-4  | 0.5569        |
| HOMO-5  | 0.4517        | HOMO-5  | 0.48          |
| HOMO-6  | 0.2931        | HOMO-6  | 0.2543        |
| HOMO-7  | 0.215         | HOMO-7  | 0.1716        |
| HOMO-8  | 0.0328        | HOMO-8  | 0.046         |
| HOMO-9  | 0.0065        | HOMO-9  | 0.0316        |

Table S6.2-4. Calculated alpha and beta frontier orbital energies of the unprotonated homocitrate S5A(H) model of E1, calculated using the BS-247 solution.

| Orbital | Energy (eV)    | Orbital | Energy (eV)    |
|---------|---------------|---------|---------------|
| LUMO    | 3.0158        | LUMO    | 2.8436        |
| HOMO    | 1.2886        | HOMO    | 1.2518        |
| HOMO-1  | 1.1796        | HOMO-1  | 1.1221        |
| HOMO-2  | 0.9418        | HOMO-2  | 1.0846        |
| HOMO-3  | 0.903         | HOMO-3  | 0.7336        |
| HOMO-4  | 0.8304        | HOMO-4  | 0.5466        |
| HOMO-5  | 0.4503        | HOMO-5  | 0.5335        |
| HOMO-6  | 0.4039        | HOMO-6  | 0.3717        |
| HOMO-7  | 0.1824        | HOMO-7  | 0.1602        |
| HOMO-8  | 0.1433        | HOMO-8  | 0.1431        |
| HOMO-9  | -0.001        | HOMO-9  | 0.1278        |

Table S6.2-5. Calculated alpha and beta frontier orbital energies of the unprotonated homocitrate S5A(H) model of E1, calculated using the BS-147 solution.
S6.3. Protonated models of E₁

S6.3.1 Protonated cluster models of E₁ in a polarizable continuum

To test the influence of the surrounding secondary environment on the energy of belt-sulfide protonated models of E₁, these models were calculated using a simple minimal cluster model of FeMoco with a surrounding polarizable continuum (\( \epsilon = 4 \)) described by CPCM. In these models the MM region and all secondary amino acid residues surrounding FeMoco are absent (homocitrate and the sidechains of His442 and Cys275 included). The resulting relative energies for these models, as well as the Fe-hydride model, is shown in Figure S6.5-1 for the BS-346 electronic state. The relative energies show that removal of the secondary coordination environment makes the bridging sulfides almost equally favorable as protonation sites.

![Figure S6.3-1](image)

**Figure S6.3-1.** BS-346 calculated energies (relative to the S2B(H) model) of the belt-sulfide protonated and Fe6 hydride E₁ models in the absence of a secondary coordination sphere.

S6.3.2 Direction of protonation in QM/MM models of E₁

It has been previously found that the direction of protonation of the FeMoco cluster embedded in a QM/MM environment can result in different relative energies. The orientations of protonation selected for each of the models in the present study was partially based on previous studies investigating the favorability of these different orientations for protonation. The protonation of the S5A position was performed such that the proton is directed towards the S3A position. The S3A position was protonated in the direction of S2B. The orientation of the proton on S2B was calculated both in the directions of S5A and S3A for the N₆-protonated state of His195; the resulting energy difference was found to be 1.4 kcal/mol, in favor of orientation towards the S3A position. Considering 1.4 kcal/mol is smaller than the uncertainty of the calculated energies in our protocol we do not think this orientation dependence is significant and
this has a small effect on the electronic and molecular structure of the cofactor, and
given the evidence of H-bonding of His195 to the S2B position in the E₀ state, the S5A oriented model was used in the EXAFS analysis. The S1A position is protonated in an endo-fashion, directed slightly towards the S2B position. Meanwhile, the S3B position is protonated in an exo-fashion nearly trans (161°) from Fe6. Lastly, hydride formation at Fe6 is positioned trans to the central carbide. The SI contains Cartesian coordinates as XYZ files of all models, showing the orientation.

S6.4. Figures & Tables

![Figure S6.4-1](image)

**Figure S6.4-1.** Enlarged version of the labeling scheme from Figure 1 of the main text.
Figure S6.4.2. The QM region used in all QM/MM models for $E_0$ and $E_1$ states.

Figure S6.4.3. Average calculated changes in the Mo-O bond distances, $\Delta R$(Mo-O), relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in bond distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0+E_1) - E_1$, and red/pink denotes $E_1 - E_0$. 
Figure S6.4-4. Average calculated changes in the Mo-S bond distances, $\Delta R(Mo-S)$, relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in bond distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0+E_1) - E_1$, and red/pink denotes $E_1 - E_0$.

Figure S6.4-5. Average calculated changes in the Mo-Fe bond distances, $\Delta R(Mo-Fe)$, relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in bond distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0+E_1) - E_1$, and red/pink denotes $E_1 - E_0$. 
Figure S6.4-6. Average calculated changes in the Fe-S bond distances, $\Delta R(\text{Fe-S})$, relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in bond distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0 + E_1) - E_1$, and red/pink denotes $E_1 - E_0$.

Figure S6.4-7. Average calculated changes in the Fe-Fe(s) bond distances, $\Delta R(\text{Fe-Fe(s)})$, relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in the Fe-Fe (short 1) scattering distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0 + E_1) - E_1$, and red/pink denotes $E_1 - E_0$. 

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Figure S6.4-8. Average calculated changes in the Fe-Fe(long) bond distances, $\Delta R(\text{Fe-Fe(l)})$, relative to each BS solution of the $E_0$ state for all BS solutions of all models and of $E_1$ in which the homocitrate is protonated. Experimentally determined changes in the Fe-Fe(long) scattering distance from EXAFS and standard errors are provided as solid lines/shaded areas spanning the width of the graph, where black/grey denotes the $(E_0+E_1) - E_1$, and red/pink denotes $E_1 - E_0$. Since the BS-235 and BS-247 solutions of the $E_0$ state produce an identical average Fe-Fe(long) distance, all $\Delta E_0(\text{BS-235})$ and $\Delta E_0(\text{BS-247})$ changes in distance appear identical for a given protonation model/BS state/His195 protonation state combination.

Figure S6.4-9. Unweighted root-mean-square deviations for the calculated changes in distances ($\Delta R_{\text{calc}}$) of the computational models of the $E_1$ state averaged across the BS-235, BS-346, and BS-247 solutions. Calculation of the unweighted RMSDs is described in the Statistical Analysis section of the Experimental details. Experimental changes in distances ($\Delta R_{\text{exp}}$) used in these calculations were acquired from $E_1-E_0$. 
Figure S6.4-10. Weighted root-mean-square deviations for the calculated changes in distances ($\Delta R_{\text{calc}}$) of the computational models of the $E_1$ state. Each individual contributing path has been weighted based on the experimental standard deviation, as described in the Statistical Analysis section of the Experimental details. Experimental changes in distances ($\Delta R_{\text{exp}}$) used in these calculations were acquired from $E_1$-$E_0$.

Figure S6.4-11. Unweighted root-mean-square deviations for the calculated changes in distances ($\Delta R_{\text{calc}}$) of the computational models of the $E_1$ state. Calculation of the unweighted RMSDs is described in the Statistical Analysis section of the Experimental details. Experimental changes in distances ($\Delta R_{\text{exp}}$) used in these calculations were acquired from $E_1$-$E_0$.

Table S6.4-1. Compilation of the calculated relative energies (both polarized QM and QM/MM energies are shown) for the various protonation models investigated, for which the $N_6$ position of His195 is protonated.

| $\Delta E$ (kcal/mol) |
|-----------------------|

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Table S6.4-2. Compilation of the calculated relative energies (both polarized QM and QM/MM energies are shown) for the various protonation models investigated, for which the $N_e$ position of His195 is protonated.

| Model | BS346 | BS235 | BS247 |
|-------|-------|-------|-------|
|       | QM    | QM/MM | QM    | QM/MM | QM    | QM/MM |
| S2B(H) | 0.4   | 0.0   | 1.2   | 1.3   | 1.4   | 2.1   |
| S3A(H) | 9.8   | 12.6  | 8.0   | 10.6  | 9.3   | 12.6  |
| S5A(H) | 0.0   | 2.6   | 1.2   | 3.6   | 2.5   | 1.1   |
| S1A(H) | 14.7  | 14.4  | 15.2  | 15.2  | 12.5  | 13.1  |
| S3B(H) | 21.5  | 23.3  | 18.3  | 21.7  | 26.3  | 26.6  |
| Fe6(H) | 10.8  | 10.4  | 14.0  | 12.8  | 13.1  | 13.3  |

Table S6.4-3. Calculated alpha and beta frontier orbital energies of the BS-235 model of E$_1$ without proton transfer, $N_{d}(H)$ protonation state of His195.

| Orbital | $\alpha$ Energy (eV) | Orbital | $\beta$ Energy (eV) |
|---------|-----------------------|---------|---------------------|
| LUMO    | 3.6474                | LUMO    | 3.6861              |
| HOMO    | 2.0627                | HOMO    | 1.8777              |
| HOMO-1  | 1.9289                | HOMO-1  | 1.8305              |
| HOMO-2  | 1.5973                | HOMO-2  | 1.73                |
| HOMO-3  | 1.5712                | HOMO-3  | 1.3393              |
| HOMO-4  | 1.3939                | HOMO-4  | 1.2155              |
| HOMO-5  | 1.1767                | HOMO-5  | 1.1357              |
| HOMO-6  | 1.1194                | HOMO-6  | 0.9645              |
| HOMO-7  | 0.9782                | HOMO-7  | 0.766               |
| HOMO-8  | 0.7133                | HOMO-8  | 0.7227              |
| HOMO-9  | 0.4961                | HOMO-9  | 0.5084              |
| HOMO-10 | 0.44                  | HOMO-10 | 0.4371              |
| HOMO-11 | 0.2834                | HOMO-11 | 0.2211              |
| HOMO-12 | 0.1977                | HOMO-12 | 0.1351              |
| HOMO-13 | 0.1304                | HOMO-13 | 0.0691              |

Table S6.4-4. Calculated alpha and beta frontier orbital energies of the BS-346 model of E$_1$ without proton transfer, $N_{d}(H)$ protonation state of His195.

| Orbital | $\alpha$ Energy (eV) | Orbital | $\beta$ Energy (eV) |
|---------|-----------------------|---------|---------------------|
| LUMO    | 3.6672                | LUMO    | 3.5565              |
| HOMO    | 2.0701                | HOMO    | 1.749               |
| HOMO-1  | 1.6714                | HOMO-1  | 1.7031              |
| HOMO-2  | 1.5545                | HOMO-2  | 1.6464              |
| HOMO-3  | 1.4308                | HOMO-3  | 1.3186              |
Table S6.4-5. Calculated alpha and beta frontier orbital energies of the BS-247 model of E₁ without proton transfer, N₅(H) protonation state of His195.

| Orbital | α       | Orbital | β       |
|---------|---------|---------|---------|
| LUMO    | 3.6417  | LUMO    | 3.6004  |
| HOMO    | 2.0671  | HOMO    | 1.8791  |
| HOMO-1  | 1.8538  | HOMO-1  | 1.8347  |
| HOMO-2  | 1.6369  | HOMO-2  | 1.7418  |
| HOMO-3  | 1.5682  | HOMO-3  | 1.3764  |
| HOMO-4  | 1.3946  | HOMO-4  | 1.26    |
| HOMO-5  | 1.2657  | HOMO-5  | 1.2181  |
| HOMO-6  | 1.0275  | HOMO-6  | 0.967   |
| HOMO-7  | 0.9775  | HOMO-7  | 0.9027  |
| HOMO-8  | 0.6587  | HOMO-8  | 0.771   |
| HOMO-9  | 0.4693  | HOMO-9  | 0.5125  |
| HOMO-10 | 0.449   | HOMO-10 | 0.4669  |
| HOMO-11 | 0.3305  | HOMO-11 | 0.2664  |
| HOMO-12 | 0.2533  | HOMO-12 | 0.1371  |
| HOMO-13 | 0.0961  | HOMO-13 | 0.0849  |

Table S6.4-6. Calculated alpha and beta frontier orbital energies of the BS-235 model of E₁ without proton transfer, N₅(H) protonation state of His195.

| Orbital | α       | Orbital | β       |
|---------|---------|---------|---------|
| LUMO    | 3.3784  | LUMO    | 3.4665  |
| HOMO    | 1.8293  | HOMO    | 1.6251  |
| HOMO-1  | 1.6833  | HOMO-1  | 1.576   |
| HOMO-2  | 1.3538  | HOMO-2  | 1.503   |
| HOMO-3  | 1.2951  | HOMO-3  | 1.1571  |
| HOMO-4  | 1.1887  | HOMO-4  | 0.9507  |
| HOMO-5  | 0.9381  | HOMO-5  | 0.8984  |
| HOMO-6  | 0.8804  | HOMO-6  | 0.7431  |
| HOMO-7  | 0.7317  | HOMO-7  | 0.5482  |
| HOMO-8  | 0.4683  | HOMO-8  | 0.4846  |
| HOMO-9  | 0.3096  | HOMO-9  | 0.2489  |
| HOMO-10 | 0.2182  | HOMO-10 | 0.2016  |
| HOMO-11 | 0.0784  | HOMO-11 | -0.029  |
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