Supporting Information

Spin polarization reveals the coordination geometry of the [FeFe] hydrogenase active site in its CO inhibited state

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1. Enzyme purification and maturation

Heterologous production of HydA1 from *Chlamydomonas reinhardtii* in *E.coli* and artificial maturation of the H-cluster were based on a previously published protocol with minor modifications. No sodium fumarate was added prior to induction of the protein expression and the purification was performed without addition of sodium dithionite. 100 mg/L ampicillin and 50 mg/L kanamycin were used as selection antibiotics throughout the expression. For the maturation the apo-protein was diluted to about 350 µM in 0.1 M Tris-HCl, 0.15 M NaCl pH 8.0. The \((\text{Et}_4\text{N})_2[\text{Fe}_2\{\text{SC}_2\text{H}_2\text{NH} \}^{(13)}\text{CN}\}_2\text{(CO)}_4]\) precursor was dissolved in DMSO and added as a 2-3 times excess and incubated for 60 min. Unbound complex was removed on a desalting column (PD-10, GE Healthcare), the additional CO was removed by shaking under a glovebox atmosphere overnight, and the maturated proteins were concentrated by ultrafiltration (Merck Millipore, Amicon Ultra-15, 30 kDa). Samples were converted to the H\text{ox}-CO state by incubation under a natural abundance CO or \(^{13}\text{CO}\) atmosphere for 30 minutes, before samples were transferred to 3 mm (o.d.) Q-band EPR tubes and frozen in liquid nitrogen, or loaded into FTIR cells.

2. FTIR spectroscopy

FTIR spectra were obtained using an IFS 66v FTIR spectrometer from Bruker Optics with a N\text{2} cooled mercury cadmium telluride (MCT) detector. Sample preparations and measurements were carried out under anaerobic conditions. Samples were placed between CaF\text{2} windows and placed in an air-tight sample holder. Spectra were recorded at 15 °C with 20 kHz velocity in double-sided forward backward mode and are the average of 1000 scans. Data processing was performed using home-written scripts in the Matlab® programming environment.
3. EPR and Pulse ENDOR spectroscopy

Field swept Q-band EPR spectra were obtained on a Bruker ELEXYS E-580 X-band spectrometer equipped with a home-built Q-band accessory with 10 W available power at 34 GHz using a home built resonator described earlier. The spectra were recorded in pulsed mode using FID detection after a 1 µs \( \pi/2 \) excitation pulse. After a pseudo-modulation transformation, the spectra obtained in this way are comparable to those obtained using CW EPR. Cryogenic temperatures (10-20 K) were obtained using a liquid helium cooled Oxford CF935 flow cryostat. ENDOR experiments were performed with the same setup making use of a water cooled 400 W Bonn-Electronic RF amplifier with frequency range 0.1 - 400 MHz (BSA 1025-400). A Trilithic\textsuperscript{TM} H4LE35-3-AA-R high-power low pass filter (cut off frequency around 35 MHz) was used to suppress the “harmonics” of the \(^1\)H ENDOR signals.

Electron nuclear double resonance (ENDOR) was used to study the \(^{13}\)C hyperfine interactions. In this investigation the Mims ENDOR as well as the Davies ENDOR sequence was used. Mims: \([\pi/2]-\tau-[\pi/2]-t_{\sigma}(RF)-t_{\sigma}-[\pi/2]-\tau-(ESE)\), Davies: \([\pi]-t_{\sigma}(RF)-t_{\sigma}-[\pi/2]-\tau-[\pi]-\tau-(ESE)\). For Mims ENDOR the excitation of nuclear spin transitions is detectable through a decrease in the ESE intensity while for Davies ENDOR the ENDOR effect is observed as an increase in intensity of the inverted spin echo. The \((\pi/2)\) pulses were set to 20 ns and the \(\tau\) delay to 200 ns. For the Davies ENDOR experiment, the initial inversion pulse was set to 100 ns. The RF pulses had a length of 60 µs. The extra delay time \(t_d\) was kept at 5 µs. For the TRIPLE experiment, the following sequence was used: Mims: \([\pi/2]-\tau-[\pi/2]-t_{\sigma}(RF_1)-t_{\sigma}(RF_2)-t_{\sigma}-[\pi/2]-\tau-(ESE)\); Davies: \([\pi]-t_{\sigma}(RF_1)-t_{\sigma}(RF_2)-t_{\sigma}-[\pi/2]-\tau-[\pi]-\tau-(ESE)\) (see Scheme S1). Here, the frequency of RF1 is fixed at a certain frequency (a specific ENDOR transition) while RF2 is swept in the usual way. As laid out in Scheme S1, these experiments allow determination of the relative signs of the HFIs of different nuclei. Absolute signs can be determined using nuclear relaxation effects such as in the “pulsed ENDOR saturation and recovery” (PESTRE)\textsuperscript{6} experiment or using a Variable Mixing Time (VMT)\textsuperscript{7} experiment. Making use of ESEEM effects in Mims ENDOR, relative signs can also be obtained without application of an additional pumping pulse.\textsuperscript{8}
Scheme S1. **Top:** Pulse sequence of the Mims TRIPLE resonance experiment. **Bottom:** Resulting signals for a system of two I=1/2 nuclei. The “ENDOR” signal is recorded with RF1 on an off-resonance frequency. The two HFI couplings (1 and 2) have opposite sign. The RF1 pulse inverts the population of the $\omega^{(2)}_{\alpha}$ transition in the $\alpha$-manifold. This reduces the population difference of the $\omega^{(1)}_{\alpha}$ transition in the same ($\alpha$) manifold but does not affect the populations in the $\beta$-manifold. In the difference TRIPLE resonance spectrum, the $\omega^{(1)}_{\alpha}$ transition appears as a weak positive contribution and the pumped $\omega^{(2)}_{\alpha}$ transition as a strong positive contribution. A similar scheme is valid for the Davies TRIPLE resonance experiment.

4. DFT calculations using the truncated model

The H-cluster was modeled using a small structural model as in our previous studies,$^9$-$^{10}$ where only the immediate ligands to the two Fe$_p$/d iron sites are included (Figure 2 in main text). In the H$_{\text{ox}}$-CO state these are four CO, two CN$^-$, the ADT (−NH−) ligand, and the cysteine side-chain modeled as CH$_3$-CH$_2$-SH thiol, with the Fe$\gamma$-bound sulfur protonated mimicking effects from the [4Fe–4Fe]$\Pi$ subcluster. The structural optimizations were done in ORCA 4.11 using the BP86 functional and a def2-TZVP basis set$^{11}$-$^{14}$ while the subsequent spin and EPR analyses was done using the B3LYP$^{15}$-$^{18}$ functional using the same basis set in which relativistic effects were accounted for using the Zero Order Regular Approximation (ZORA)$^{19}$. The calculations include atom pairwise dispersion correction$^{20}$-$^{21}$. The SOMO properties and the spin population representation in Figure 4 (main text) were generated using ChemCraft.$^{22}$
To investigate the effect of Hartree-Fock (HF) exchange on the calculated HFIs, the EPR calculations were performed using functionals with different amounts of HF-exchange: BP86 (0%), TPSSh (10%), B3LYP manually set to 15%, and B3LYP using the default 20% HF-exchange (see Tables S1 and S2).

Table S1. EPR parameters of the truncated model S-COa (see main text) using functionals with variable Hartree Fock (HF) exchange. The experimental g-values and $^{13}$C-HFI interactions were obtained in the current study while the $^{57}$Fe HFI for Fe$_p$ and Fe$_d$ were obtained from reference 23. HFIÆs are given in MHz.

| Method | EXP | BP86 | TPSSh | B3LYP | B3LYP |
|--------|-----|------|-------|-------|-------|
| HF-exchange | % | 0% | 10% | 15% | 20% |
| g1     | 2.008 | 2.0028 | 2.0036 | 2.0050 | 2.0057 |
| g2     | 2.009 | 2.0123 | 2.0121 | 2.0149 | 2.0162 |
| g3     | 2.058 | 2.0156 | 2.0167 | 2.0220 | 2.0237 |
| g$_{iso}$ | 2.025 | 2.0102 | 2.0108 | 2.0140 | 2.0152 |
| A$_{iso}$-Fe$_p$ | 4.4 | +3.12 | +5.09 | +3.90 | +3.97 |
| A$_{iso}$-Fe$_d$ | 1.3 | -3.33 | -1.51 | -1.93 | -1.30 |
| A$_{iso}$-$^{13}$CN$_1$ | (-)7.3 | -18.18 | -23.68 | -25.25 | -27.27 |
| A$_{iso}$-$^{13}$CN$_2$ | (-)4.0 | -9.47 | -14.68 | -14.96 | -17.33 |
| A$_{iso}$-$^{13}$CO$_{ext}$ | (+)17.1 | +49.49 | +35.51 | +37.20 | +31.82 |

Table S2. EPR parameters of the truncated model S-COb (see main text) using functionals with variable Hartree Fock (HF) exchange. The experimental g-values and $^{13}$C-HFI interactions were obtained in the current study while the $^{57}$Fe HFI for Fe$_p$ and Fe$_d$ were obtained from reference 23. HFIÆs are given in MHz.

| Method | EXP | BP86 | TPSSh | B3LYP | B3LYP |
|--------|-----|------|-------|-------|-------|
| HF-exchange | % | 0% | 10% | 15% | 20% |
| g1     | 2.008 | 2.0004 | 2.0021 | 2.0035 | 2.0047 |
| g2     | 2.009 | 2.0097 | 2.0084 | 2.0107 | 2.0113 |
| g3     | 2.058 | 2.0130 | 2.0142 | 2.0189 | 2.0207 |
| g$_{iso}$ | 2.025 | 2.0077 | 2.0083 | 2.0110 | 2.0122 |
| A$_{iso}$-Fe$_p$ | 4.4 | +2.76 | +5.858 | +4.75 | +5.30 |
| A$_{iso}$-Fe$_d$ | 1.3 | -4.23 | -1.24 | -2.25 | -1.36 |
| A$_{iso}$-$^{13}$CN$_1$ | (-)7.3 | -17.84 | -23.41 | -4.92 | -26.84 |
| A$_{iso}$-$^{13}$CN$_2$ | (-)4.0 | +43.93 | +35.52 | +37.60 | +34.35 |
| A$_{iso}$-$^{13}$CO$_{ext}$ | (+)17.1 | -9.69 | -12.67 | -13.11 | -14.21 |

All calculations predict negative HFIs for the basal $^{13}$CN Æ ligands (indicated in blue) and a positive HFI for the apical $^{57}$CO (indicated in red). The calculations also consistently predict a negative isotropic HFI for $^{57}$Fe$_d$ although both iron have a positive spin population (Figure 3 in main text). This can be explained since the calculated spin populations at the iron core are dominated by the d-orbitals which have no density at the iron nucleus. The $^{57}$Fe isotropic HFI is determined by the s-orbital population which is only a fraction of the total and can be negative. The carbons of the basal CO/CN Æ ligands have no direct spin density from the SOMO (see Figure 3 main text). However, one can assume that the Ï-bonds to the Fe core are polarized by the spin density in the Fe-d$_{xy}$ type orbitals of the SOMO much like the situation in
aromatic organic radicals where the sigma bonds of the protons are polarized by the magnetic $\pi$-orbitals.\textsuperscript{24}

The g-values as well as the $^{57}$Fe-HFI interactions are best reproduced by the B3LYP calculations. All functionals overestimate the magnitude of the $^{13}$C HFIs but the ratio between $^{13}$CN$_{ap}$, $^{13}$CN$_{ad}$, and $^{13}$CO$_{ext}$ is best reproduced in the BP86 calculation (with no HF-exchange). It should be noted, however, that the DFT method, in general, has serious and unintelligible deficiencies especially for treatment of open shell TMC systems and in the estimation of Fermi contact interactions.\textsuperscript{25} The best and most stable results are obtained using a combination of a hybrid functional (such as B3LYP), a sufficiently large basis set (def2-TZVP) and relativistic corrections.\textsuperscript{25} As will be shown below, this level of theory provides a reasonable approximation of the electronic structure of the iron core (i.e. g-parameters and $^{57}$Fe-HFIs) also for the extended models including Broken Symmetry treatment.

5. DFT calculations using extended models

In order to investigate the effect of the [4Fe-4S]$_{II}$ subcluster on the spin distribution and EPR parameters of the H-cluster in the H$_{50}$CO state, we performed calculations on large models L-COa (Figure S1) and L-COb including the cubane cluster. The coordinating cysteines were modeled as S-CH$_3$ moieties. The EPR parameters were calculated for both the BP86 and the B3LYP functional as listed in Table S3. For all calculations, the broken symmetry configuration BS1 was selected since the L-COa model as well as the models including protein side chains (see below) automatically converge into this configuration. The L-COb model does not converge in this configuration. Therefore, for the EPR property calculations, the BS1 configuration was explicitly imposed during the calculations. For model “LCOb-opt” the H-cluster was separately geometry optimized in the BS1 configuration.

Table S3. EPR parameters of the extended models L-COa (apical CO$_{ext}$) and L-COb (basal CO$_{ext}$) including the [4Fe-4S]$_{II}$ sub-cluster in the BS1 broken symmetry configuration (Figure S1). The “LCOb-opt” model was geometry optimized in the BS1 configuration. The experimental g-values and $^{13}$C-HFI interactions were obtained in the current study while the $^{57}$Fe HFI for Fe$_{p}$ and Fe$_{d}$ were obtained from reference.\textsuperscript{23} HFIs are given in MHz.

| Method      | EXP | L-COa | L-COb | LCOb-opt |
|-------------|-----|-------|-------|----------|
| HF-exchange |     |       |       |          |
| g1          | 2.008 | 2.001 | 1.480 | 2.007 | 0.309 | 1.999 | 0.767 |
| g2          | 2.009 | 2.026 | 1.562 | 2.029 | 2.768 | 2.022 | 1.500 |
| g3          | 2.058 | 2.045 | 2.179 | 2.050 | 3.855 | 2.034 | 11.57 |
| g-iso       | 2.025 | 2.024 | 1.740 | 2.029 | 2.310 | 2.019 | 4.612 |
| A$_{iso}$-Fe$_{1}$ | 4.4 | 3.48 | -2.33 | +3.97 | -6.51 | +3.53 | -56.45 |
| A$_{iso}$-Fe$_{2}$ | 1.3 | -1.83 | +2.76 | -1.47 | -18.11 | -1.84 | -5.42 |
| A$_{iso}$-$^{13}$CN$_{1}$ | (-)7.3 | -21.89 | -14.33 | -20.37 | -13.55 | -20.71 | -14.08 |
| A$_{iso}$-$^{13}$CN$_{2}$ | (-)4.0 | -19.96 | -9.15 | +36.90 | +42.43 | +36.24 | +43.39 |
| A$_{iso}$-$^{13}$CO$_{ext}$ | (+)17.1 | +35.32 | +46.48 | -18.08 | -9.51 | -53.38 | -25.02 |

For the B3LYP calculations the obtained EPR parameters are not fundamentally different from those obtained using the truncated models (S-COa and S-COb) as presented in Table S1 and S2. Clearly, the
BP86 calculation completely fails to predict the correct g-values and $^{57}$Fe-HFI. On the other hand, the HFI values for the $^{13}$CO/$^{13}$CN' ligands are comparable to those obtained for the truncated models S-COa and S-COb.

To assess the effect of the protein surrounding, we also ran calculations including the directly coordinating amino acid side chains as well as parts of the protein that have a steric interaction with the CN/CO ligands on the Fe$_d$ side (Ser232, Cys299, Lys358, Pro324 in Figure 1). Model FP1-COa contains the selected amino acid side chains. For model PF2-COa the protein surrounding is removed without geometry optimization. For model PF3-COa the positions of the coordinating CH$_3$-S carbons of the cubane were fixed while the binuclear subcluster was geometry optimized. In this process the bond between the bridging thiol and Fe$_p$ was elongated from 2.4 to 2.75 Å. In Table S4 the obtained EPR parameters are listed. The signs and magnitude of the $^{13}$CO/$^{13}$CN' ligands are consistent with those obtained with the S-COa and L-COa models. Except that, interestingly, the magnitudes of the Fe$_p$ and Fe$_d$ HFI s and, concomitantly, those of $^{13}$CN$_p$ and $^{13}$CN$_d$, have been reversed. Removing the protein surrounding with its H-bonds to the CN' ligands (model FP2) only has a small effect on the HFI magnitudes. Geometry optimization of [2Fe]$_4$ (model FP3) restores the order observed for models S-COa and L-COa (included in Table S4 for comparison). Apparently, elongation of the bond between the bridging thiol and Fe$_p$ has a strong effect on the spin population distribution between Fe$_p$ and Fe$_d$. Experimentally, the HFI of Fe$_p$/Fe$_d$ and CN$_p$/CN$_d$ cannot be distinguished. The current assignment is based on the S-CO and L-CO models. Selective $^{13}$CO labeling$^{26}$ in combination with ENDOR/ESEEM studies may be feasible and could resolve this ambiguity.

![Figure S1. Model L-COa including the [4Fe-4S]$_{14}$ sub-cluster. The red arrows indicate the Broken Symmetry spin configuration (BS1) selected for the irons in the cubane cluster.](image-url)
Table S4. EPR parameters of the extended models FPn-COa (where n = 1, 2 or 3) including the [4Fe-4S]_H subcluster in the BS1 broken symmetry configuration as well as amino acids in the protein surrounding: Ser232, Cys299, Lys358, Pro324 (Figure 1). The experimental g-values and isotropic \(^{13}\text{C}\)HFI interactions were obtained in the current study while the \(^{57}\text{Fe}\) HFI for Fe_p and Fe_d were obtained from reference 23. HFIs are given in MHz. FP1: full protein surrounding. FP2: H-cluster frozen but surrounding removed. FP3: cubane frozen, [2Fe]_H geometry optimized.

|                  | Exp  | B3LYP: FP-COa |
|------------------|------|---------------|
|                  |      | FP1 | FP2 | FP3 | L-COa |
| \(g_x\)          | 2.008| 1.98 | 1.99 | 2.002 | 2.008 |
| \(g_y\)          | 2.009| 2.013| 2.017| 2.010 | 2.0264 |
| \(g_z\)          | 2.058| 2.033| 2.038| 2.043 | 2.0449 |
| \(\Delta_{\text{iso}}\)_{\text{Fe_p}}\)  | 4.4  | -0.73 | 0.01 | +3.86 | +3.48 |
| \(\Delta_{\text{iso}}\)_{\text{Fe_d}}\)  | 1.3  | -4.84 | -4.29 | -1.63 | -1.83 |
| \(\Delta_{\text{iso}}\)_{\text{CN_p}}\)  | -7.3 | -13.1 | -14.5 | -21.2 | -21.89 |
| \(\Delta_{\text{iso}}\)_{\text{CN_d}}\)  | -4.0 | -26.1 | -26.7 | -20.5 | -19.96 |
| \(\Delta_{\text{iso}}\)_{\text{CO_ext}}\) | +17.1| +58.9 | +54.7 | +38.3 | +35.32 |
Figure S2 Top: FTIR of $\text{H}_{\text{ox}}\text{-CO}$ in which both CN ligands are exchanged with $^{13}\text{CN}$ (2048 and 2040 cm$^{-1}$). The proximal CO has its stretch at 1963 cm$^{-1}$ while the distal CO and the extraneous CO stretches are coupled (2011 and 1969 cm$^{-1}$). Bottom: FTIR of $\text{H}_{\text{ox}}\text{-CO}$ using extraneous $^{13}\text{CO}$. It can be seen that the CO$_d$ and CO$_{ext}$ stretches are “uncoupled” leaving CO$_{ext}$ at 1991 cm$^{-1}$ and CO$_d$ at 1944 cm$^{-1}$ as indicated by the arrows.
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