

## SUPPORTING INFORMATION

for

### Artificially Maturated [FeFe] Hydrogenase from *Chlamydomonas reinhardtii*: A HYSCORE and ENDOR Study of a Non-Natural H-cluster

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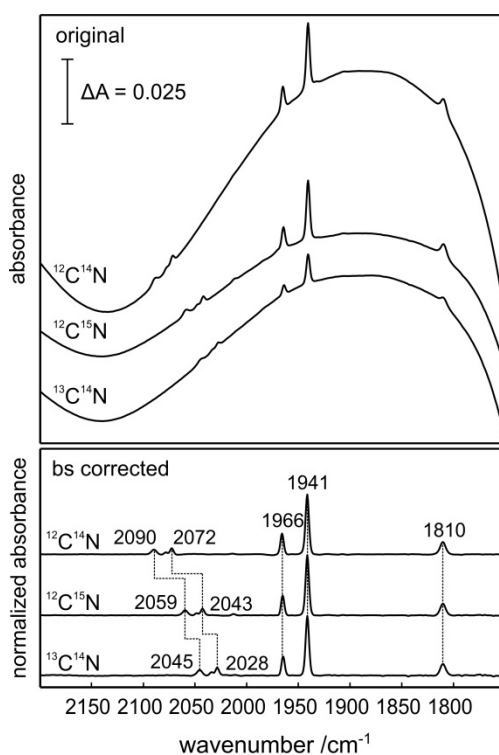


Figure S1. FTIR absorbance spectra and the baseline corrected spectra recorded for oxidized CrHydA1(pdt) containing non-labeled and two labeled pdt complexes.

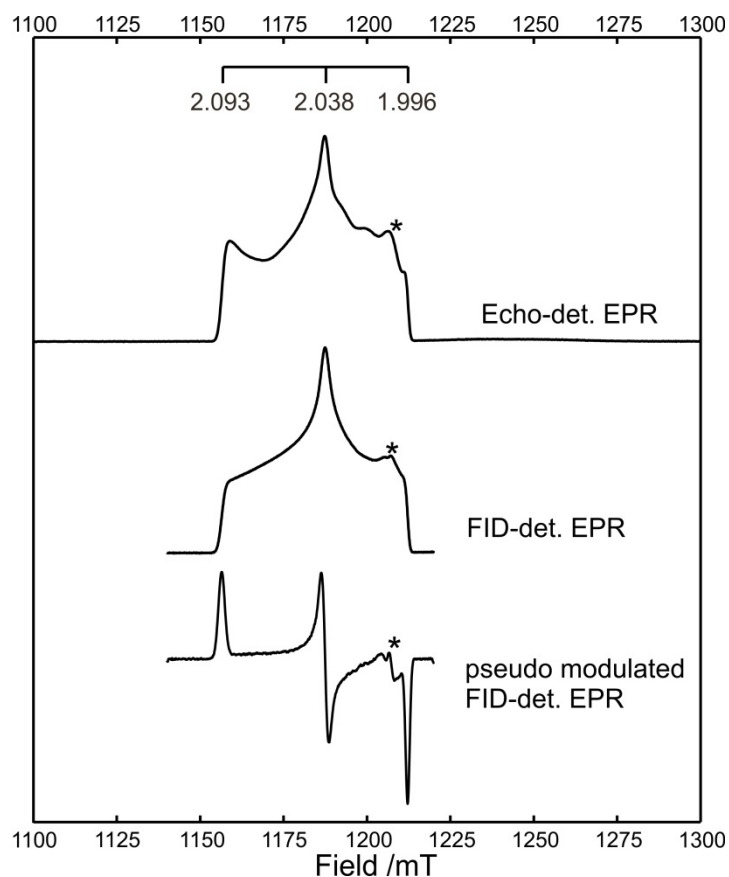


Figure S2. Q-band 2-pulse detected EPR (top), FID detected EPR (middle) and first derivative of FID-detected EPR (bottom) spectra of oxidized CrHydA1(pdt) measured at 20 K. The 2-pulse detected EPR spectrum was measured with microwave pulse lengths  $\pi/2 = 16$  ns and  $\pi = 32$  ns and the separation between the two pulses was  $\tau = 500$  ns. The shot repetition time was 1 ms and the microwave frequency 33.868 GHz. The FID detected EPR spectrum was measured with a microwave pulse length of 1  $\mu$ s, shot repetition time 1 ms, microwave frequency 33.868 GHz. Above the spectra g-values obtained from simulation are presented. The asterisk indicates an overlapping signal originating from a small amount of [3Fe-4S] cluster.

The 2-pulse echo detected EPR spectrum is affected by the field dependent phase memory time and nuclear spin modulations originating from interactions with  $^{14}\text{N}$  nuclei. To avoid such distortion FID-detected EPR spectra were recorded for each sample.

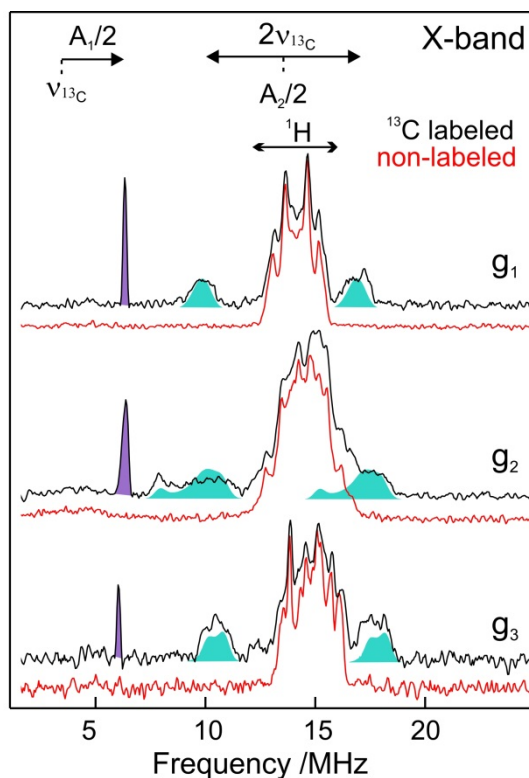


Figure S3. X-band Davies ENDOR spectra measured for non-labeled (red) and  $^{13}\text{C}$  labeled  $\text{CN}^-$  ligands (black) of oxidized CrHydA1(pdt) at 15 K at the field positions corresponding to  $g_1$ ,  $g_2$  and  $g_3$ . All spectra were recorded using an RF pulse of 20  $\mu\text{s}$  and a shot repetition time of 2 ms. The microwave frequency for measurement on the labeled sample was 9.716 GHz and the field positions: 330.9 mT ( $g_1$ ), 339.7 mT ( $g_2$ ), 346.8 mT ( $g_3$ ). For the non-labeled sample a microwave frequency of 9.716 GHz was used at field positions: 331 mT ( $g_1$ ), 339.8 mT ( $g_2$ ), 346.9 mT ( $g_3$ ). The assignments of the hyperfine couplings are indicated at the top of the figure.

The most prominent signal in the X-band Davies ENDOR spectra (see figure S3) is centered at 14.5 MHz and is also present in the non-enriched sample. This  $^1\text{H}$  ENDOR signal with maximum splitting of 3.5 MHz is very similar to the one obtained for native  $\text{H}_{\text{ox}}$  and was assigned to the  $\beta$ -protons of the cysteine ligands coordinating the  $[\text{4Fe-4S}]_{\text{H}}$  sub-cluster.

## Calculation of the angle between $^{13}\text{C}$ and $^{15}\text{N}$ hyperfine tensors

To obtain angles between two hyperfine tensors first the rotation matrixes were calculated (using EasySpin in Matlab). EasySpin is using the following convention for the definition of Euler angles:

$$\begin{aligned}
 R &= R_z(\gamma) \cdot R_y(\beta) \cdot R_x(\alpha) \\
 &= \begin{pmatrix} \cos\gamma & \sin\gamma & 0 \\ -\sin\gamma & \cos\gamma & 0 \\ 0 & 0 & 1 \end{pmatrix} \cdot \begin{pmatrix} \cos\beta & 0 & -\sin\beta \\ 0 & 1 & 0 \\ \sin\beta & 0 & \cos\beta \end{pmatrix} \cdot \begin{pmatrix} \cos\alpha & \sin\alpha & 0 \\ -\sin\alpha & \cos\alpha & 0 \\ 0 & 0 & 1 \end{pmatrix} \\
 &= \begin{pmatrix} \cos\gamma\cos\beta\cos\alpha - \sin\gamma\sin\alpha & \cos\gamma\cos\beta\sin\alpha + \sin\gamma\cos\alpha & -\cos\gamma\sin\beta \\ -\sin\gamma\cos\beta\cos\alpha - \cos\gamma\sin\alpha & -\sin\gamma\cos\beta\sin\alpha + \cos\gamma\cos\alpha & \sin\gamma\sin\beta \\ \sin\beta\cos\alpha & \sin\beta\sin\alpha & \cos\beta \end{pmatrix}
 \end{aligned}$$

Subsequently, the relative angles between the two tensor axis frames can be calculated by evaluating the inproduct matrix of the two rotation matrices as shown below:

The rotation matrix for the  $^{13}\text{C}$  hyperfine tensor rotated around  $\alpha = 0$ ,  $\beta = 119$  and  $\gamma = 46$  is calculated as:

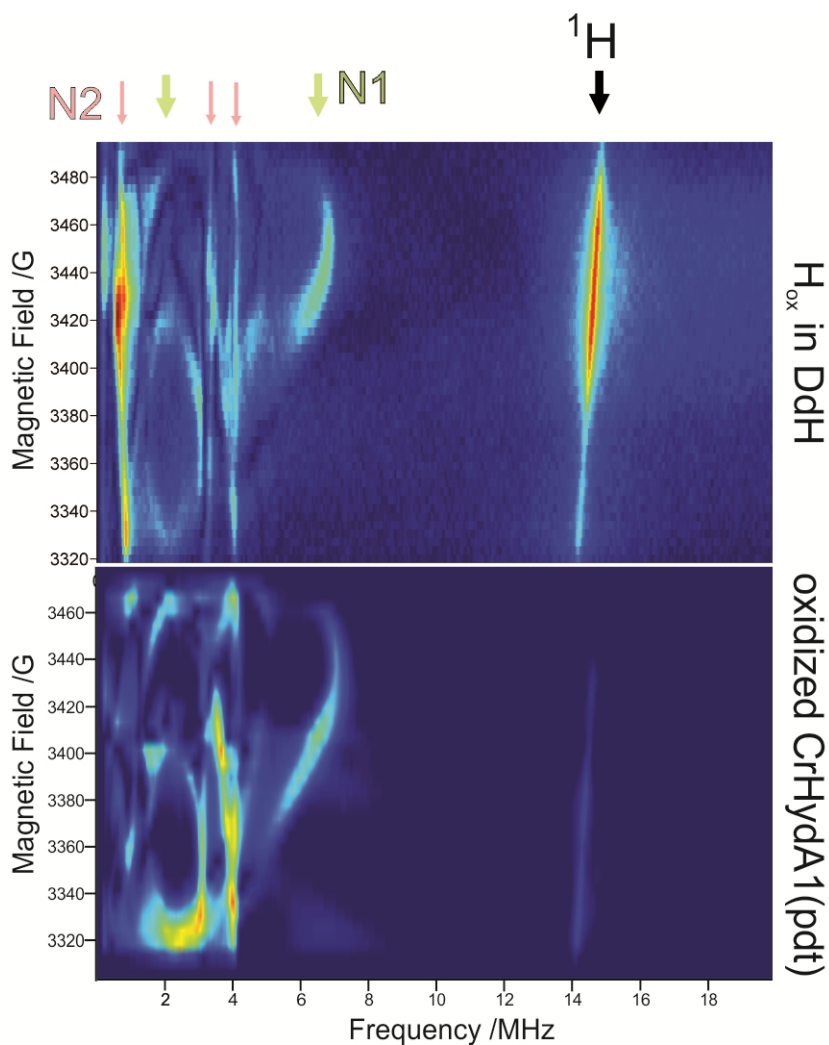
$$R_{^{13}\text{C}} = \begin{pmatrix} -0.3368 & 0.7193 & -0.6076 \\ 0.3487 & 0.6947 & 0.6291 \\ 0.8746 & 0 & -0.4848 \end{pmatrix}$$

For the rotation matrix for the  $^{15}\text{N}$  hyperfine tensor rotated around  $\alpha = 0$ ,  $\beta = 50$  and  $\gamma = 90$  we obtain:

$$R_{^{15}\text{N}} = \begin{pmatrix} 0 & 1 & 0 \\ -0.6428 & 0 & 0.7660 \\ 0.7660 & 0 & 0.6428 \end{pmatrix}$$

The matrix representing the relative angles in degree between the axes of these two tensors equals to:

$$\text{angles} = \begin{pmatrix} 63.5 & 116.5 & 140.9 \\ 109.7 & 44 & 127.4 \\ 33.9 & 57.8 & 80.2 \end{pmatrix}$$



**Figure S4.** Comparison of the X-band 3-pulse ESEEM vs magnetic field of oxidized CrHydA1(pdt) (bottom) with the previously published H<sub>ox</sub> state from DdH (top)<sup>1</sup>. Both spectra were measured under the same experimental conditions: temperature 15 K, length of microwave pulses  $\pi/2 = 8$  ns, delay between first two pulses  $\tau = 180$  ns. Microwave frequency was 9.726 GHz (for the oxidized CrHydA1(pdt)) and 9.778 GHz (for the H<sub>ox</sub> state from DdH).

The large difference in intensity between the <sup>1</sup>H signals is most likely a result of the overlapping additional signal from H<sub>ox</sub>-CO present in the native DdH preparation. In both spectra a <sup>14</sup>N signal is present showing a strong field dependence; it is assigned to nitrogen from the CN<sup>-</sup> ligand.

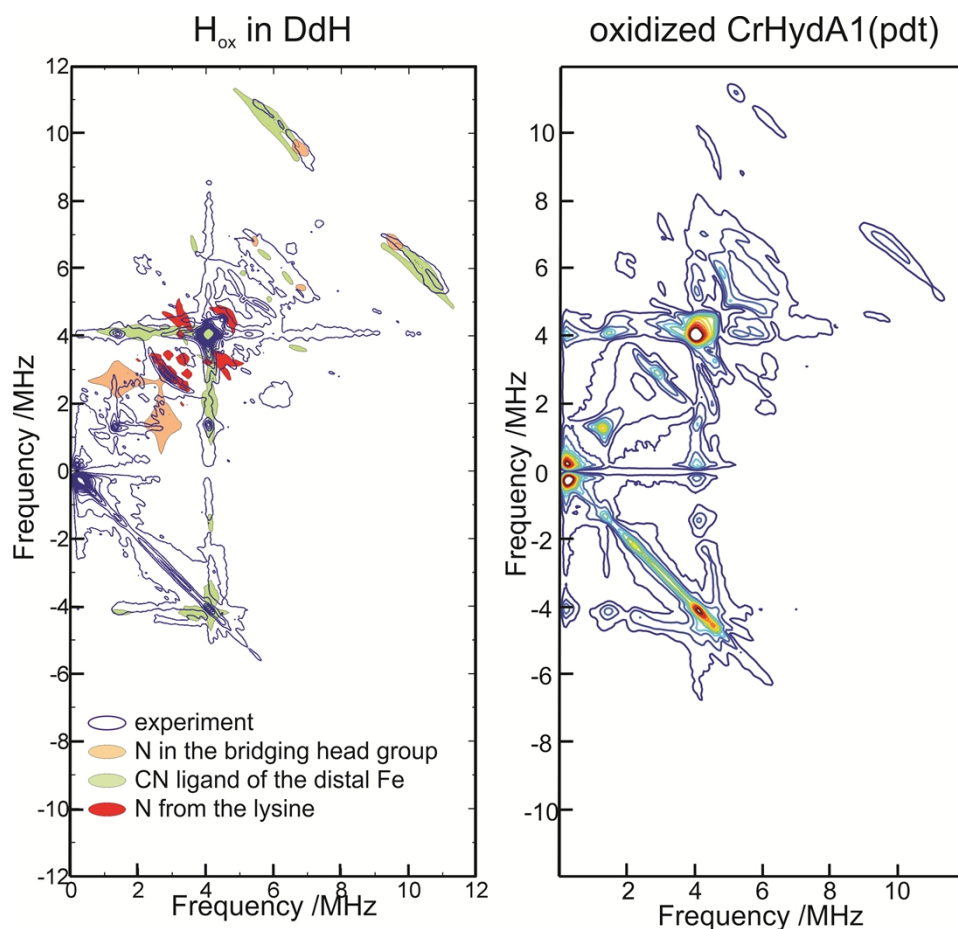


Figure S5. Comparison of the Q-band HYSCORE spectra of oxidized CrHydA1(pdt) (right) with the previously published  $H_{ox}$  state from DdH (left) measured at a field corresponding to the  $g_2$  position<sup>1</sup>. Both spectra were measured under the same experimental conditions: temperature 20 K,  $t_1$  and  $t_2$  step 16 ns, shot repetition time 500  $\mu$ s, delay between the two first microwave pulses ( $\tau$ ) 268 ns. Microwave frequency was 33.867 GHz (for the oxidized CrHydA1(pdt)) and 33.865 GHz (for the  $H_{ox}$  state from DdH). The length of microwave pulses previously used was ( $\pi/2$ ) 36 ns and 16 ns in the current measurements.

A signal previously assigned to the nitrogen in the bridging head group is not present in the HYSCORE spectrum obtained for oxidized CrHydA1(pdt).

#### Reference List

- (1) Silakov, A.; Wenk, B.; Reijerse, E.; Lubitz, W. *Physical Chemistry Chemical Physics* **2009**, *11*, 6592.