Electronic Supplementary Information for

Serine is the molecular source of the $NH(CH_2)_2$ bridgehead moiety of the *in vitro* assembled [FeFe] hydrogenase H-cluster

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Content

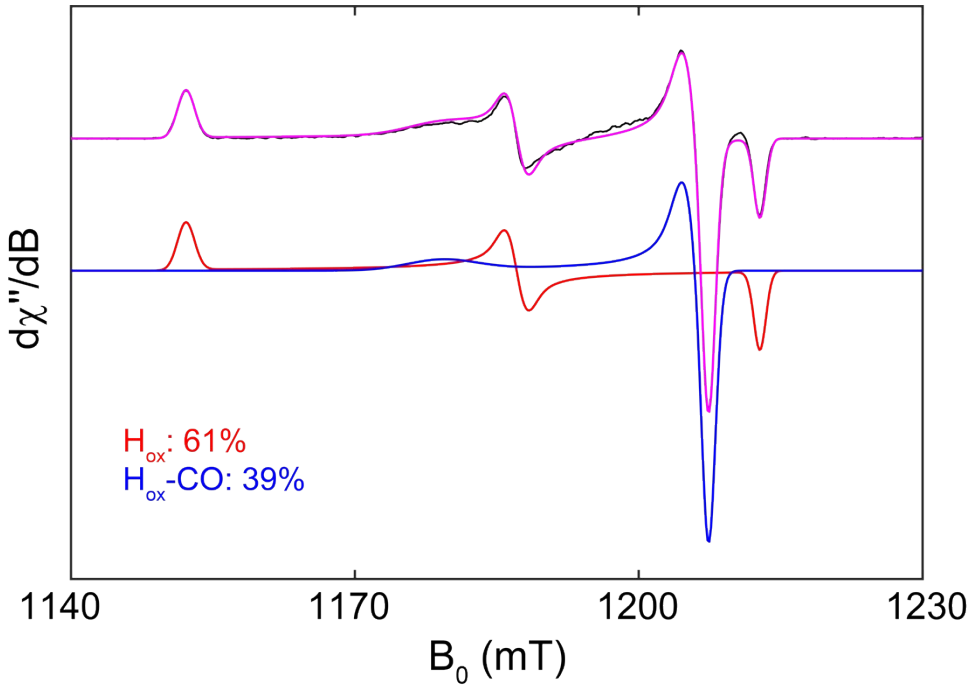


Figure S1. Q-band pseudomodulated echo-detected EPR spectrum of a typical *Cr*HydA1 sample isolated from the maturation reaction supplemented with labeled amino acids and oxidized with 2 mM thionine (black trace). The spectrum is simulated with two components: 61% H_{ox} (red trace) and 39% H_{ox}-CO (blue trace). The ratio between the H_{ox} and H_{ox}-CO is based on double integration. Total simulation is shown in magenta trace. Conditions: frequency = 33.9 GHz; temperature = 15 K; τ = 300 ns; modulation amplitude = 1 mT. Simulation parameters for the two components are: H_{ox}, *g* = [2.1033, 2.0414, 1.9981], HStrain = [60, 65, 38] MHz; H_{ox}-CO, *g* = [2.0555, 2.0099, 2.0074], HStrain = [250, 75, 42] MHz.

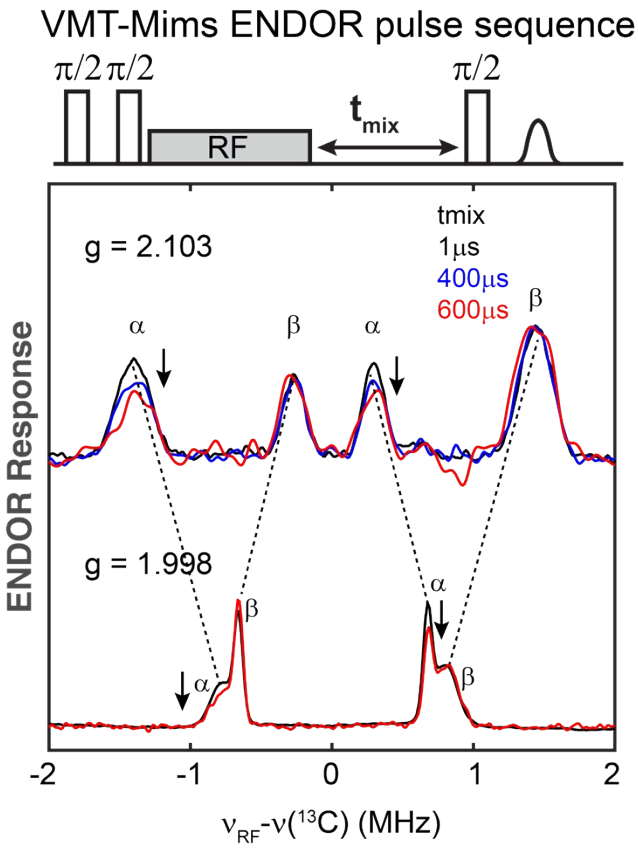


Figure S2. Various mixing time ^{13}C Mims ENDOR spectra of [^{13}C, ^{15}N-adt]-HydA1 recorded at two field positions. For the outer ^{13}C signal with the hyperfine coupling of +[3.40, 1.35, 1.37] MHz, the relative intensity of the low RF transition decreases as t_{mix} increases, which is characteristic of the corresponding nuclear spin-flip transition being between levels within the α electron-spin manifold. The sign of this hyperfine tensor is therefore positive. In contrast, the hyperfine sign for the inner ^{13}C signal can be determined to be negative. In addition to determining the sign of the hyperfine couplings, VMT Mims ENDOR performed at different field positions also helps to deconvolute the two sets of ENDOR signals.

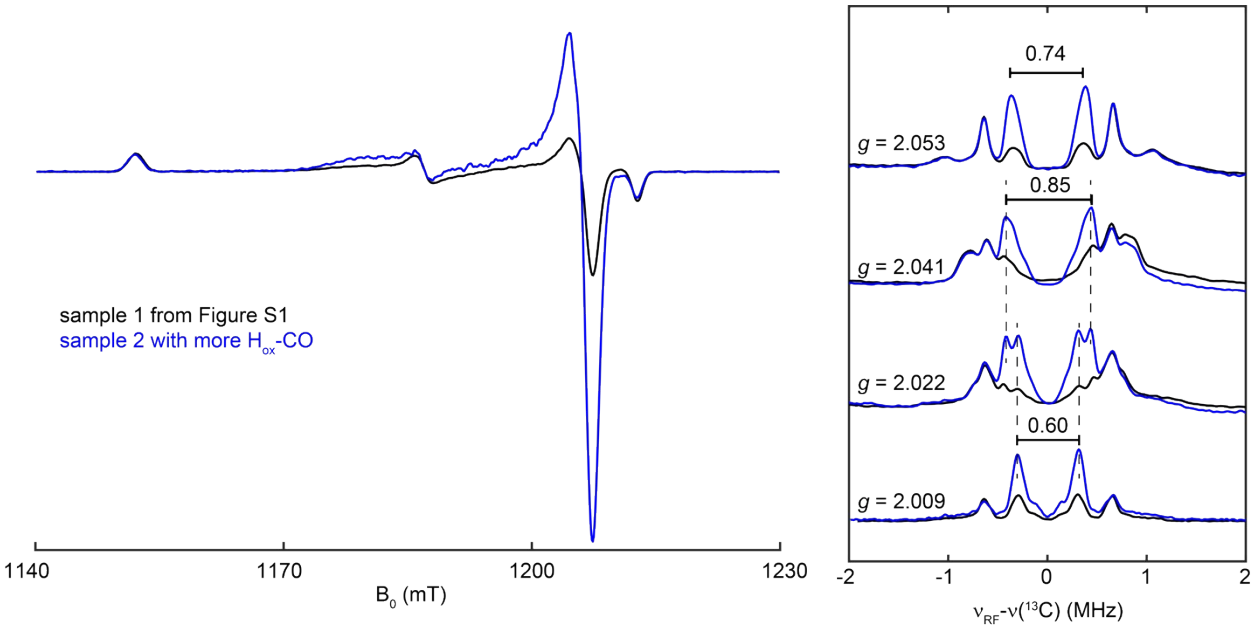


Figure S3. Analysis of the ^{13}C Mims ENDOR spectra of adt ^{13}C atoms in H_{ox}-CO. The Mims ENDOR spectra of [^{13}C, ^{15}N-adt]-HydA1 is complicated by the presence of H_{ox}-CO at field positions from $g \sim 2.055$ to $g \sim 2.007$. To deconvolute the contribution of H_{ox}-CO, the EPR spectra (left) and field-dependent ^{13}C Mims ENDOR spectra (right) of two samples are shown in Figure S3, one from Figure S1 (black traces) and the other with much higher H_{ox}-CO ratio (blue traces). The two sets of spectra are normalized to the contribution from H_{ox}. It is then obvious that the ^{13}C ENDOR signals with hyperfine couplings from 0.60-0.85 MHz can be attributed to H_{ox}-CO adt ^{13}C atoms. Similar to the scenario for H_{ox}, the two ^{13}C atoms in the adt ligand of H_{ox}-CO likely have different hyperfine tensors. However, these two ^{13}C hyperfine tensors are overlapped with each other. Spectral simulation was not attempted to further extract these parameters with higher precision.

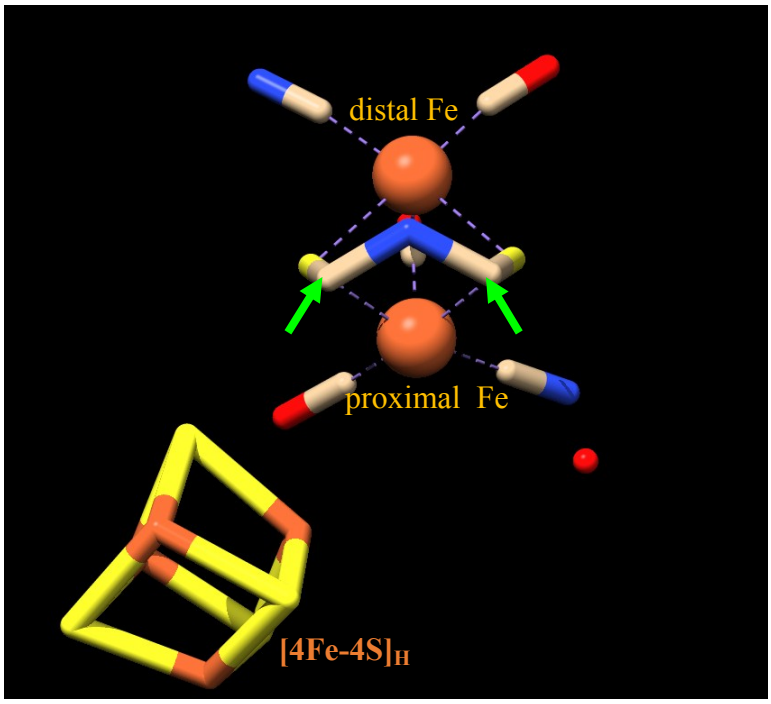
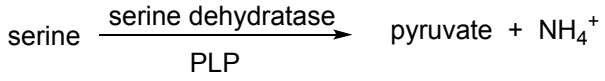
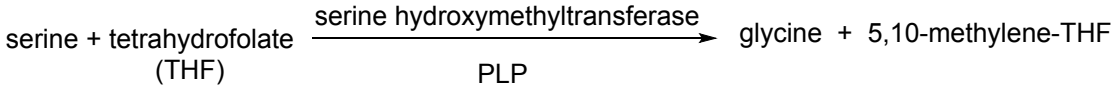


Figure S4. Top view of the H-cluster (PDB ID code: 4XDC[1]). The two carbon atoms in the adt ligand are highlighted by the green arrow. The distal iron site is asymmetrically coordinated by CO and CN^- ligands. The presence of the $[4Fe\text{-}4S]_H$ subcluster also makes the two adt carbons geometrically inequivalent.

Further discussion on the role of Ser

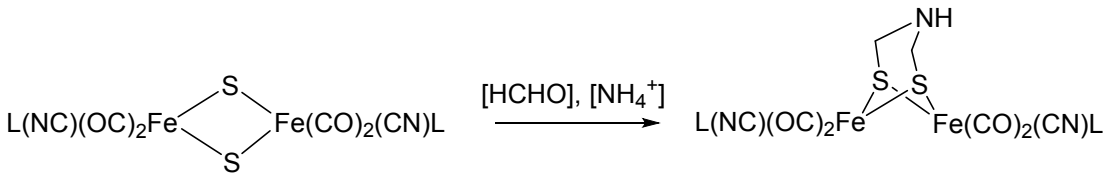
If Ser is involved in the PLP-dependent process, we identified two such enzymes in *E. coli* common metabolic pathways: the serine dehydratase and the serine hydroxymethyltransferase. The reactions catalyzed by these two enzymes are as following:





The reaction catalyzed by serine hydroxymethyltransferase is particularly interesting because it serves as the major source of the one-carbon unit available to cells,[2] and it is consistent with the C3 methylene group of Ser being transferred. This reaction essentially transfers a biological formaldehyde molecule onto THF. However, this reaction alone does not explain how the C-N-C connectivity in the CH_2NHCH_2 fragment is formed. In addition, as shown in Figure 2, when ^{13}C, ^{15}N-glycine, which is the product of serine hydroxymethyltransferase, is supplemented in the maturation reaction, the nitrogen atom in the adt ligand is not labeled with ^{15}N, suggesting that this is not the reaction responsible for the formation of the NH fragment.

In our previous study using the synthetic $[Fe(Cys)(CO)_2(CN)]$ carrier in the H-cluster maturation, we found that the Cys S atom is transferred into the adt ligand, with the rest of Cys converted into pyruvate.[3] Cleavage of the C-S bond in Cys leaves behind an iron-sulfide, as shown below. It is possible that the adt ligand is formed on the sulfide from a biological source of HCHO or HCHO equivalent and a biological source of NH_4^+, in reactions similar to those used in the synthesis of the $[Fe_2(adt)(CO)_4(CN)_2]$ complex by Rauchfuss et al:[4]



In this regard, both serine dehydratase and serine hydroxymethyltransferase could be involved in the metabolism of Ser and the formation of adt by catalyzing the reactions of generating biological NH_4^+ and biological HCHO, respectively. We think this is a possible explanation as to how Ser donates both its amino group and 3-CH_2 group to form the adt ligand. Notably, while Ser is the major source of one-carbon metabolism, other such sources in cells may also contribute to the biosynthesis of the adt ligand.

References

1. Esselborn, J.; Muraki, N.; Klein, K.; Engelbrecht, V.; Metzler-Nolte, N.; Apfel, U. P.; Hofmann, E.; Kurisu, G.; Happe, T., A structural view of synthetic cofactor integration into [FeFe]-hydrogenases. *Chemical Science* **2016,** *7* (2), 959-968.
2. Bailey, L. B., *Folate in Health and Disease*. CRC Press: Boca Raton, 2009.
3. Rao, G.; Pattenaude, S. A.; Alwan, K.; Blackburn, N. J.; Britt, R. D.; Rauchfuss, T. B., The binuclear cluster of [FeFe] hydrogenase is formed with sulfur donated by cysteine of an [Fe(Cys)(CO)2(CN)] organometallic precursor. *Proc. Natl. Acad. Sci. U. S. A.* **2019,** *116* (42), 20850-20855.
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