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The radical SAM enzyme HydE generates adenosylated Fe(I) intermediates en route to the [FeFe]-hydrogenase catalytic H-cluster

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Abstract:

The H-cluster of [FeFe]-hydrogenase consists of a [4Fe–4S]_H-subcluster linked by a cysteinyl bridge to a unique organometallic [2Fe]_H-subcluster assigned as the site of interconversion between protons and molecular hydrogen. This [2Fe]_H-subcluster is assembled by a set of Fe–S maturase enzymes HydG, HydE and HydF. Here we show that the HydG product [Fe^{II}(Cys)(CO)₂(CN)] synthon is the substrate of the radical SAM enzyme HydE, with the generated 5'-deoxyadenosyl radical attacking the cysteine S to form a C5'–S bond concomitant with reduction of the central low-spin Fe(II) to the Fe(I) oxidation state. This leads to the cleavage of the cysteine C3–S bond, producing a mononuclear [Fe^I(CO)₂(CN)S] species that serves as the precursor to the dinuclear Fe(I)Fe(I) center of [2Fe]_H-subcluster. This work unveils the role played by HydE in the enzymatic assembly of the H-cluster, and expands the scope of radical SAM enzyme chemistry.

Introduction

The [FeFe]-hydrogenase enzyme, which efficiently catalyzes the reversible interconversion of H_2 and H^+/e^- , is important for microbial metabolism and serves as a model catalyst for the production and the use of hydrogen as a renewable fuel.¹ Its catalytic H-cluster (Fig. 1) consists of a canonical $[4Fe-4S]_H$ -subcluster linked to an organometallic $[2Fe]_H$ -subcluster through a cysteine (Cys) residue.² There is much interest in understanding how nature biosynthesizes the $[2Fe]_H$ -subcluster, in which the two Fe centers are coordinated by one bridging-azadithiolate ligand and multiple CO/ CN^- ligands. Three Fe-S “maturase” enzymes, HydG, HydE and HydF, play central roles in this biosynthesis.³⁻⁵ The radical *S*-adenosyl-L-methionine (rSAM) enzyme HydG is currently the best understood: it lyses its substrate tyrosine using rSAM reactivity initiated at one 4Fe-4S cluster and then installs the resultant tyrosine-derived CO and CN^- onto a unique fifth “dangler” Fe of an auxiliary Fe-S cluster, ultimately forming its organometallic product, a $[Fe^{II}(Cys)(CO)_2(CN)]$ synthon which is vectored into the further synthesis of the $[2Fe]_H$ -subcluster.⁶⁻¹⁰ HydF has been proposed to serve as a scaffold in assembling the $[2Fe]_H$ -subcluster and delivering it to the apo-hydrogenase.¹¹⁻¹² Relatively little is actually known about the chemistry carried out by HydE, the other rSAM enzyme of the H-cluster maturase family. HydE has been hypothesized to play a role in building the bridging-azadithiolate ligand of the H-cluster, and assays of SAM turnover have been interpreted to favor a possible thiol-containing substrate.¹³ In addition, Nicolet et al. have shown that HydE can react with the unnatural substrate 1,3-thiazolidine to form a new C5'-S bond.¹⁴ Although this reaction has no direct biochemical significance to the generation of the H-cluster, it is relevant to the present report, where we have now identified a specific biosynthetic function for HydE in this important bioassembly process.

[FeFe]-hydrogenase can be produced via cell-free synthesis (or *in vitro* maturation) using the HydG, HydE, and HydF proteins delivered in their isolation lysates,¹⁵ a cocktail of small molecule components along with an apo-hydrogenase HydA1 (*Chlamydomonas reinhardtii*) which is expressed with only the $[4Fe-4S]_H$ -subcluster of the H-cluster.¹⁵ In this process, HydG, the native source of the $[Fe^{II}(Cys)(CO)_2(CN)]$ synthon assembly precursor, is absolutely required for generating active hydrogenase enzyme. However, we have recently shown that the H-cluster in [FeFe]-hydrogenase can be installed through *in vitro* maturation without the HydG enzyme, by instead including a synthetic “syn-B” complex in the maturation media.¹⁶ On the basis of elemental

analysis, syn-B appears to a cluster consisting of 3~4 $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})(\text{H}_2\text{O})]$ centers bound to a high-spin $\text{Fe}(\text{II})$.¹⁶ The key feature of syn-B complex is that it allows active hydrogenase maturation with only two maturase enzymes, HydE and HydF, i.e., it serves as a fully functional carrier of HydG's $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ product. This new semisynthesis route affords us the ability to initiate the *in vitro* maturation with isotopically-labeled cysteine, or alternatively selenocysteine, synthetically preinstalled in the $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ synthon unit of syn-B.¹⁶ This approach allowed us to use pulse electron paramagnetic resonance (EPR) spectroscopy, extended X-ray absorption fine structure (EXAFS), and mass spectrometry to show that the two Fe-bridging sulfurs of the bridging-azadithiolate of the $[\text{2Fe}]_{\text{H}}$ -subcluster are sourced from the cysteine sulfur of the synthon (or its selenium atoms if using selenocysteine).¹⁶ However, the central C-N-C moiety of the bridging-azadithiolate is not supplied by this cysteine, but is instead sourced from serine, specifically serine's C3 and the amino N.¹⁷

The isotope labeling, combined with isotope sensitive spectroscopy, has allowed us to determine the molecular sourcing of all the atoms of the H-cluster (Fig. 1), but questions remain about the underlying enzymatic mechanisms for the steps beyond the HydG generation of the $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ synthon. How might the organometallic synthon be further tailored, and how might two such low-spin ferrous centers be brought together to make the binuclear cluster? To answer these questions one possible approach is to explore the reactivity of the HydG product synthon with the downstream maturation enzymes (HydE and/or HydF). While such attempts are largely impeded by the instability of the synthon product of HydG, we anticipate that employing the synthetic syn-B compound as a surrogate should facilitate our understanding of the later stages in the maturation pathway.

Herein, we report the reactivity of the rSAM enzyme HydE with selected isotopologs of the HydG product $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ unit as provided by the synthetic syn-B complex. We have identified that the role of rSAM enzyme HydE in the $[\text{FeFe}]$ -hydrogenase H-cluster bioassembly is to enzymatically modify the HydG product $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ synthon by reducing the central $\text{Fe}(\text{II})$ to the $\text{Fe}(\text{I})$ oxidation state via a radical-addition mechanism that produces adenosylated $\text{Fe}(\text{I})$ intermediates. This reduction is followed by disassembling the Fe-bound cysteinyl ligand to generate an $\text{Fe}^{\text{I}}\text{S}(\text{CN})(\text{CO})_2$ entity, which we propose serves as the precursor to the dinuclear $\text{Fe}(\text{I})\text{Fe}(\text{I})$ center of $[\text{2Fe}]_{\text{H}}$ -subcluster.

Results

HydE contains a typical rSAM [4Fe–4S] cluster binding motif (CX₃CX₂C) near its N-terminus.^{18–20} Such a [4Fe–4S] cluster is generally used to generate a highly reactive 5'-dA• radical via the reductive cleavage of a coordinated SAM. The canonical rSAM reaction employs this “hot” 5'-dA• radical to abstract an H-atom from the enzyme's given substrate, such as the case of HydG where a hydrogen atom in the tyrosine amino group is abstracted as the first step in fragmenting tyrosine and generating CO and CN[–].^{6–7, 18, 21} Some HydE enzymes, such as those from *Thermotoga maritima* and *Clostridium acetobutylicum*, also contain an accessory Fe–S cluster binding motif (CX₇CX₂C) at the C-terminus (Fig. S1). However, this C-terminal cluster is not conserved (e.g., not present in *Shewanella oneidensis*, *Bacteroides thetaiotaomicron*, or *Desulfovibrio desulfuricans*)²² and is not essential for the maturation of active [FeFe]-hydrogenase.¹⁹

To explore possible HydE reactivity with the synthetic [Fe^{II}(Cys)(CO)₂(CN)] carrier, HydE-reaction mixture was prepared by mixing purified *Thermotoga maritima* HydE enzyme (*TmHydE*, with its accessory cluster deleted by mutation as it has no reported function and provokes local structural heterogeneity),^{14, 19} along with the reductant sodium dithionite (DTH), SAM, and the syn-B complex. This reaction mixture was incubated for varying times before freeze-quenching in liquid nitrogen (*see* Methods). We then used EPR spectroscopy to detect and characterize any resulting paramagnetic intermediates.

Characterization of a 10 s mononuclear low-spin Fe(I) intermediate. As shown in Fig. 2A, freeze-quenching after a time of 10 s produces a single paramagnetic species with an *S* = 1/2 rhombic EPR spectrum (35 K) with a *g* tensor of [2.093, 2.050, 2.003]. This signal is distinct from that of the reduced SAM-bound [4Fe–4S]⁺ cluster (Fig. 2B) of *TmHydE*, which has a *g* tensor of [2.019, 1.882, 1.840], and which can only be detected at lower temperature (10 K) due to its intrinsic rapid spin-relaxation. Control experiments (Fig. 2A) show that all four components, *TmHydE* enzyme, DTH, SAM and syn-B, are required for generating this novel paramagnetic species, indicating that syn-B is reacting with *TmHydE* through a HydE-mediated rSAM reaction. Moreover, this reaction is both HydE and syn-B specific, as no paramagnetic intermediates are generated when we replaced *TmHydE* with another rSAM enzyme, HydG, or when we employed another Fe(II) compound that carries a [Fe^{II}(cysteamine)(CO)₂(CN)] unit instead of [Fe^{II}(Cys)(CO)₂(CN)] (Fig. 2C). Observation of this 10 s paramagnetic intermediate shows that

the [Fe^{II}(Cys)(CO)₂(CN)] synthon unit donated by syn-B is a functional substrate for the rSAM chemistry mediated by *TmHydE*.

The rhombic **g** tensor for 10 s-intermediate corresponds to an isotropic component $g_{\text{iso}} = 2.049$ ($g_{\text{iso}}^2 = (g_1^2 + g_2^2 + g_3^2)/3$), which deviates significantly from the value $g_e = 2.0023$ for a free electron. The *g*-value deviation, proportional to the one-electron spin-orbit coupling constant,²³ suggests that the 10 s-intermediate signal is metal-centered, presumably a species resulting from HydE's reaction with the [Fe^{II}(Cys)(CO)₂(CN)] substrate. We can rule out an Fe(II) origin, which would be either low-spin diamagnetic ($S = 0$) or integer high-spin ($S = 2$) with a dramatically different EPR spectrum, likely unobservable in our conventional perpendicular-polarization X-band EPR measurement.²⁴ We considered a low-spin Fe(III) ($3d^5$, $S = 1/2$) origin for the signal. However, due to the very significant spin-orbit coupling effects, low-spin Fe(III) species typically offer dramatically different **g** tensors with a much larger g_{max} ($\gtrsim 3.0$) and much broader overall spectral lineshapes.^{23, 25} These considerations indicate that the 10 s-intermediate is a low-spin Fe(I) ($3d^7$, $S = 1/2$) species. The **g** tensor of this 10 s-intermediate is quite similar to that of the H_{ox} state of the H-cluster (*g*-values = [2.103, 2.042, 1.998]), in which the [2Fe]_H-subcluster is poised in a mixed-valence Fe(II)Fe(I) state with most of the spin density carried by the Fe(I) site distal to the [4Fe-4S]_H-subcluster.²⁶ Also, the 10 s-intermediate's **g** tensor is close to that of a synthetic low-spin mononuclear Fe(I) compound with a side-on acetylene ligand ($S = 1/2$, *g*-values = [2.114, 2.040, 2.007]).²⁷ Therefore, the EPR spectra and analyses suggest that the 10 s-intermediate corresponds to a low-spin Fe(I)-containing species resulting from a HydE mediated one-electron reduction of the Fe(II) center of the synthon unit.

To further probe the structure of the 10 s-intermediate, we generated its isotopologues using syn-B containing ¹³CN⁻ or ¹³C₃/¹⁵N-Cys ligands. Hyperfine couplings to these magnetic isotopes were probed using the pulse EPR technique of ENDOR (Electron Nuclear Double Resonance) (Fig. 3). The Q-band ¹³C Davies-ENDOR spectra of the 10 s-intermediate generated with syn-B-¹³CN (Fig. 3A) shows one set of strongly-coupled ¹³C with $A = [28.5, 22.0, 29.5]$ MHz, a hyperfine coupling comparable to the strongly-coupled ¹³C of ¹³CN assigned to the distal Fe(I) center of the H_{ox} state of the H-cluster.²⁶ Also, the corresponding X-band CW EPR spectrum (Fig. S2) shows a ¹³C-split doublet at g_3 , analogous to the splitting seen in the ¹³CN-labeled H_{ox} state CW EPR signal.²⁶ In addition, only this strongly-coupled ¹³C is observed, and no weaker-coupled ¹³C such as that assigned to the ¹³CN of the proximal Fe(II) of the H-cluster can be detected (Fig.

S3). These ENDOR results further support our *g*-tensor based Fe(I) assignment to the 10 s-intermediate, and also suggest that it is an Fe(I) monomer since a mixed valence Fe(II)Fe(I) species similar to the [2Fe]_H-subcluster in the H_{ox} state would likely show a second ENDOR signal from a more weakly-coupled ¹³CN. This identification of the 10 s Fe(I) monomer intermediate confirms that the [Fe^{II}(Cys)(CO)₂(CN)] synthon unit carried by syn-B is successfully delivered to the HydE enzyme and converted to a new species, revealing that the organometallic [Fe^{II}(Cys)(CO)₂(CN)] synthon product of HydG is the substrate for HydE.

The ¹³C₃/¹⁵N-Cys isotopologue of syn-B was used to examine if the cysteine ligand is also present in the 10 s-intermediate. Here two sets of ¹³C hyperfine-couplings ([1.05, 1.50, 1.38] MHz and [0.42, 0.98, 0.96] MHz), as well as one set of ¹⁵N couplings ([5.41, 5.69, 4.23] MHz), are detected with Q-band pulse ENDOR spectroscopy (Fig. 3B), demonstrating that the cysteine introduced in the [Fe^{II}(Cys)(CO)₂(CN)] synthon unit remains as a ligand to the Fe(I) center of the 10 s-intermediate.

A striking result was obtained when the 10 s-intermediate was generated using ¹³C₁₀-adenosine-labeled SAM (Fig. 3C). ENDOR experiments reveal a ¹³C hyperfine coupling ([6.0, 4.8, 8.2] MHz) between the Fe(I) spin-center and one ¹³C of the ¹³C₁₀-adenosine-labeled SAM, indicating a linkage between the Fe(I) center and the adenosine moiety of SAM. This ¹³C coupling is stronger than the ¹³C couplings from the cysteine ligated via amino and thiol groups but weaker than that of the directly coordinated ¹³CN. The ¹³C coupling is also weaker than that reported for the “Omega” organometallic intermediate where the C5' of adenosine is directly coordinated to an Fe of the 4Fe–4S cluster.²⁸ Theoretical calculations (*see* DFT results in Fig. S4 & S5) suggest this ~6.0 MHz ¹³C coupling reasonably arises from the C5' covalently linked to the Cys sulfur atom, as shown in Fig. 3C, and as preceded by the prior study that shows the 5'-dA• radical generated by *Tm*HydE directly attacks the sulfur atom of an unnatural substrate, 1,3-thiazolidine, forming a new C5'–S bond.¹⁴

In this case with syn-B providing the [Fe^{II}(Cys)(CO)₂(CN)] synthon, such a linkage would be possible if the 5'-dA• attacks the electron-rich sulfur atom of Cys in [Fe^{II}(Cys)(CO)₂(CN)] to form a new C5'–S bond. Radical addition to sulfur would be accompanied by the one-electron reduction of the Fe(II) center to Fe(I), consistent with the collective results described above (Fig. 5). To confirm this proposal we turned to high-performance liquid chromatography–mass

spectrometry. While it was not possible to detect the fully intact 10 s organometallic intermediate, we succeeded in detecting the proposed organic *S*-adenosyl-L-cysteine adduct, with appropriate mass patterns found in reactions carried out using $^{13}\text{C}_{10}$ -adenosine-labeled SAM, syn-B- $^{13}\text{C}_3/^{15}\text{N}$ -Cys or syn-B-selenocysteine (Fig. S6). These results confirm the presence of the *S*-adenosyl-L-cysteine moiety and reinforce our proposed mechanism invoking 5'-dA• radical addition to cysteine sulfur.

These results establish the first-stage in the radical-initiated reduction of the low-spin Fe(II) of the $[\text{Fe}(\text{Cys})(\text{CO})_2(\text{CN})]$ synthon to Fe(I) en route to H-cluster formation and installation in HydA1. This builds upon the prior work of Nicolet et al.¹⁴ showing that the 5'-dA• radical generated by *TmHydE* directly attacks the sulfur atom of 1,3-thiazolidine to form a new C5'-S bond. We now show a similar reaction occurs in HydE's interaction with syn-B, which we have previously shown to be a functional donor of the organometallic $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ precursor on path to hydrogenase activation.¹⁶ Here the 5'-dA• radical reacts directly with the synthon's cysteine sulfur to form a C5'-S bond. This reinforces that point first made by Nicolet et al.¹⁴ that HydE is a member of very small family of rSAM enzymes in which the generated 5'-dA• radical does not drive hydrogen-atom abstraction, but instead performs another function, such as addition of the 5'-dA• radical to a double bond to generate a C-C bond.²⁹⁻³⁰

The further time evolution of the Fe(I) intermediate. We investigated the evolution of the HydE reaction with syn-B at longer times, obtaining EPR spectra (35 K observation temperature) of reaction mixtures quenched at 2 min and 10 min (Fig. 4A). The X-band CW EPR spectrum of the 2 min-reaction sample recorded at 35 K exhibits a rather complicated signal with at least two components (Fig. S7), while at 10 min the EPR spectrum is dominated by a single new $S = 1/2$ signal with a **g** tensor of $[g_1, g_2, g_3] = [2.098, 2.080, 2.005]$ ($g_{\text{iso}} = 2.061$) that is amenable for further pulse EPR interrogation. Although the central g_2 value is shifted appreciably relative to the 10 s-intermediate, the total span of g -values and its spin-relaxation properties (with the optimal signal acquired at 35 K), are similar to those of the 10 s-intermediate, suggesting that this species trapped at 10 min is also a low-spin Fe(I) species. The 10 min sample prepared with syn-B- ^{13}C N shows only one ^{13}C hyperfine coupling ($A \approx 27$ MHz, Fig. 4B), as well as the clear ^{13}C -split doublet presented at the g_3 position of the corresponding CW EPR spectrum (Fig. S7), again indicating that at 10 min in the reaction the intermediate remains a mono-Fe(I) species with one CN ligand.

But in stark contrast to the 10 s-intermediate, the 10 min-species prepared with syn-B- $^{13}\text{C}_3/^{15}\text{N}$ -Cys shows no sign of ^{13}C or ^{15}N couplings to the Fe(I) center. GC-MS of the reaction mixture show the presence of $^{13}\text{C}_3$ -labeled pyruvate ($\sim 100\%$ isotope enriched, *see* SI Methods), indicating that the cysteine C3–S bond has been cleaved. Thus HydE is now shown responsible for the on-pathway reaction characterized in our prior work with syn-B in the full *in vitro* maturation experiment,¹⁶ in which the Fe–S bond is retained while the entire C/N backbone of Cys is cleaved away, ultimately to be replaced by the serine-derived C–N–C bridgehead moiety.¹⁷

For the 10 min-species prepared with $^{13}\text{C}_{10}$ -adenosine-labeled SAM, we observe a ~ 1.5 MHz ^{13}C hyperfine coupling (Fig. 4B), showing that adenosine ^{13}C remains coupled to the Fe(I) center and indicating that the C5'–S bond remains intact at the 10 min time point. Although the $^{13}\text{C}5'$ hyperfine value is smaller than that of 10 s-intermediate, this could be due to less spin-density delocalized to C5' resulting from a possible geometric reorganization with the release of the C/N-backbone of Cys (*see* DFT in Fig. S8). We also performed the full maturation reaction using $^{13}\text{C}_{10}/^{15}\text{N}_5$ -adenosine-labeled SAM and characterized the resulting H_{ox} H-cluster signal by ENDOR (Fig. S9), with no hyperfine couplings detected, showing that the ribose linkage must be released before or concomitant with the installation of the final C–N–C bridgehead.

Discussion

Experimental characterization of the 10 s and 10 min species of the HydE syn-B unit reaction positions us for future experiments to determine how HydE, HydF, and HydA1 complete the biosynthesis of the $[\text{2Fe}]_{\text{H}}$ -subcluster. In the meantime, we can speculate: the generation of Fe(I) centers by HydE should be a prerequisite for the formation of an Fe(I)Fe(I) dimer, of which there are a number of synthetic examples.^{31–33} By comparing the geometric structure of the 10 min-species with the $[\text{2Fe}]_{\text{H}}$ -subcluster of the H-cluster, we can envision a possible dimerization process (Fig. 5) that is coupled to the installation of the serine-derived bridging $\text{NH}(\text{CH}_2)_2$. Probing this chemistry is our next experimental target using our combination of chemical and isotope labeling, pulse EPR methods, and other spectroscopies.

In summary, by employing the synthetic syn-B complex in reaction time-dependent EPR experiments, we have established that the $[\text{Fe}^{\text{II}}(\text{Cys})(\text{CO})_2(\text{CN})]$ HydG-reaction product, as donated by our synthetic carrier syn-B, is the substrate of the HydE enzyme. Our prior work shows this reaction is on pathway to H-cluster formation and activation of hydrogenase activity. This new

work provides important new details of the enzymology of HydE, the second rSAM enzyme employed in the process. We show that HydE reduces the low-spin Fe(II) center of the synthon to generate a mononuclear low-spin Fe(I) intermediate via a radical-addition mechanism. The as-formed Fe(I) intermediate further evolves to release the C/N-containing fragment of Cys to form a mononuclear $[\text{Fe}^{\text{I}}(\text{CO})_2(\text{CN})\text{S}]$ species which is well suited to condense to a $[\text{Fe}_2^{\text{I}}(\text{CO})_4(\text{CN})_2\text{S}_2]$ center, reminiscent of the $\text{Fe}_2(\text{CO})_6\text{S}_2$ species of Hieber and Gruber.³⁴ This compact inorganic species needs only the installation of the C-N-C bridgehead to complete the $[\text{2Fe}]_{\text{H}}$ -subcluster. Therefore, this work reveals a new role for rSAM enzymes in formation of an unusual organometallic Fe(I) species and provides new direct experimental insights in the role played by HydE in the enzymatic assembly of the H-cluster.

Methods.

Preparation of syn-B complex. Synthetic organometallic chemistry leading to the syn-B complexes employed in this work, including **syn-B**, **syn-B-¹³CN**, **syn-B-¹³C₃/¹⁵N-Cys**, **syn-B-cysteamine** and **syn-B-selenocysteine**, was conducted by using glovebox and Schlenkline techniques. Detailed descriptions of syn-B synthesis are given in reference.¹⁶ The corresponding Fourier transform infrared (FT-IR) spectra as well as the electrospray ionization–mass spectrometry (ESI-MS) characterization are also provided by reference.¹⁶

Expression and purification of *TmHydE* enzyme. The plasmid used in this work is a C-terminal *strep*-tag II containing pET-21b(+) vector with a codon-optimized *Thermotoga maritima hydE* gene insert in which the three C-terminal cysteine (Cys) residues are mutated to serine residues (*C311S/C319S/C321S*). This mutant is based on previous work by Nicolet et al.^{14, 19} which suggested that the semi-conserved accessory Fe–S cluster has no reported function for the [FeFe]-hydrogenase H-cluster activity and provokes a local structural heterogeneity. Therefore, the *TmHydE* enzyme employed in this work contains only the N-terminal rSAM [4Fe–4S] cluster, simplifying EPR assignments. We also confirmed that the wild-type *TmHydE* enzyme generates the same mononuclear Fe(I) intermediate at 10 s (shown in Fig. S10).

The plasmid was transformed into *E. coli* BL21(DE3) Δ *iscR* competent cell with kanamycin resistance. Cells were grown in Luria-Bertani broth containing 40 mg/L kanamycin, 100 mg/L ampicillin, 2 mM ammonium ferric citrate, 0.5% (w/v) glucose and 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS, pH = 7.8) at 28 °C to an O.D.₆₀₀ \approx 0.4. Then the cultures were pooled, transferred into the anaerobic chamber, and supplemented with 5 mM Cys and 10 mM fumarate. The pooled culture was stirred in the chamber for *ca.* 40 min to deplete the remaining oxygen in the solution, and protein expression was then induced by 0.25 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). After \sim 22 h, cells were harvested by centrifugation (6,000 rpm at 4 °C for 30 min), flash frozen in liquid nitrogen and stored at -80 °C.

For protein purification, cells were lysed in a HEPES buffer (buffer W, 50 mM HEPES, 150 mM KCl, pH = 8.0) containing 1 \times Bugbuster detergent solution (EMD Millipore), 25 U/mL benzonase (EMD Millipore), 1 kU/mL rLysozyme (EMD Millipore), and one EDTA-free protease inhibitor cocktail tablet (Roche). The cell debris was removed by centrifugation (18,000 rpm at 4 °C for 30 min) and the clear supernatant was loaded to a 50 mL column volume of *strep*-

tactin resin in a gravity column. The unbound protein fraction was removed by gravity-flowing through the resin and the resin was washed with 100 mL buffer W. The *TmHydE* protein fraction was eluted by adding 100 mL buffer W containing 3 mM desthiobiotin. The dark protein fraction was collected. The as-eluted protein with the concentration of $\sim 300\ \mu\text{M}$ was aliquoted, flash frozen in liquid nitrogen and stored at $-80\ ^\circ\text{C}$.

EPR sample preparation. *TmHydE* reaction mixtures were prepared anaerobically. 100 μL as-eluted *TmHydE* ($\sim 300\ \mu\text{M}$) in a pH-buffered solution (50 mM HEPES, 150 mM KCl, pH = 8.0) was incubated with 20 equivalents of sodium dithionite (DTH) and 10 equivalents of SAM for ~ 10 min. Then around 10 equivalents of syn-B aqueous solution was added to initiate the reaction. After being allowed to react for set times, samples were frozen and stored in liquid N_2 for EPR characterization. We confirmed that the organometallic Fe(I) species can also be generated by *SoHydE* (see Fig. S11), with faster kinetics compared to the thermophile *TmHydE*.

Supporting Information Available: Detailed experimental methods; DFT calculations of the Fe(I) intermediates generated through HydE reaction; LC-MS analysis of HydE reaction samples; similar Fe(I) intermediate generated by *SoHydE* from *Shewanella oneidensis*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Data and materials availability: All data are available in the manuscript or the supplementary materials.

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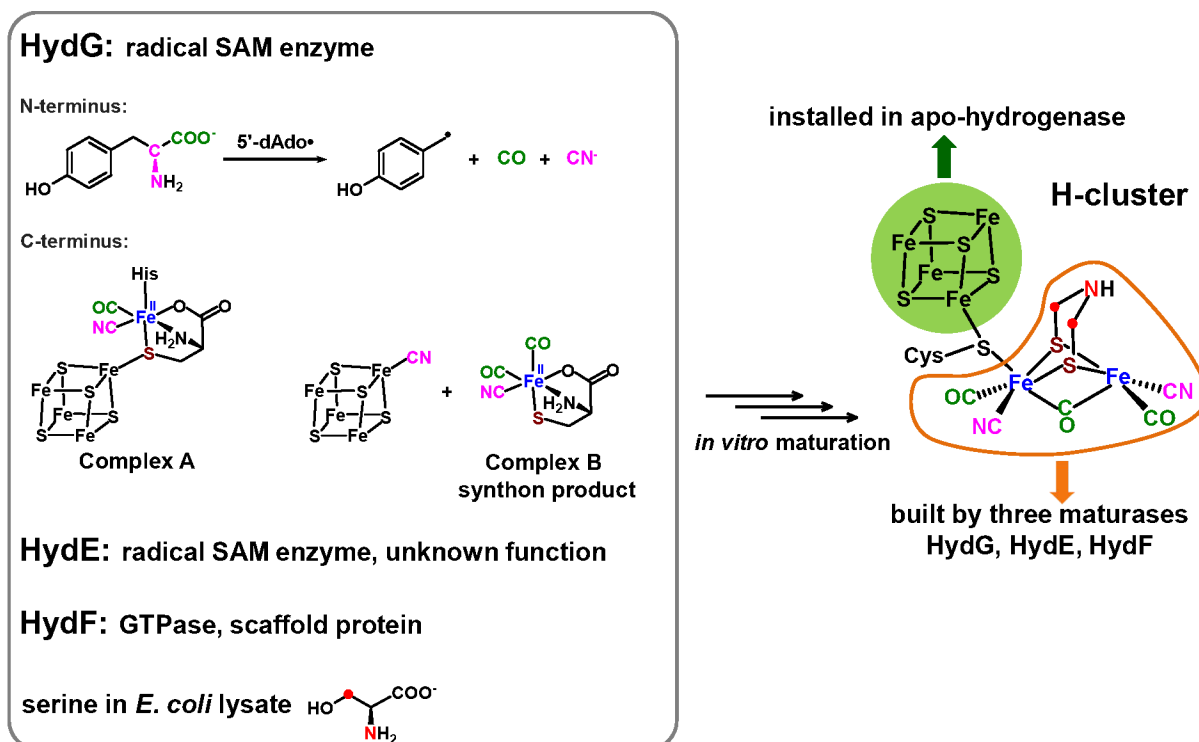


Fig. 1 (double column). The cell-free *in vitro* maturation of [FeFe]-hydrogenase H-cluster requires three maturation enzymes: HydG, HydE and HydF, in addition to the HydA1 apo-enzyme that is expressed with only the [4Fe-4S] component. Alternatively, the H-cluster can be built by substituting the HydG enzyme with a synthetic synthon-complex “syn-B” that delivers the HydG-reaction product, a [Fe^{II}(Cys)(CO)₂(CN)] synthon.¹⁶ The colors displayed in the [2Fe]_H-subcluster correspond to the molecular sourcing, including the bridging-azadithiolate sulfurs (in burgundy) which come from the cysteine of the synthon,¹⁶ and the C-N-C moiety (in red) which is sourced from serine (C3 and amino N).¹⁷

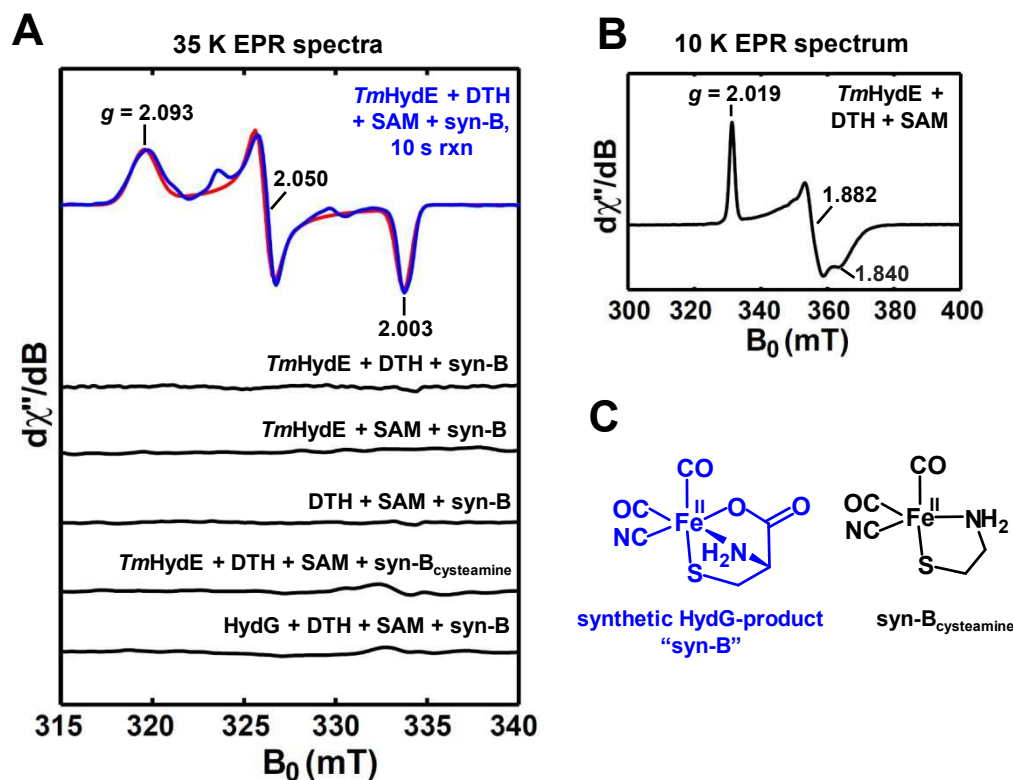


Fig. 2 (double column). (A) X-band (9.37 GHz, 35 K) CW EPR spectrum (blue trace) of the *TmHydE* reaction mixture prepared by incubating *TmHydE* enzyme with 20 equivalents of DTH, 10 equivalents of SAM and 10 equivalents of syn-B for 10 s before flash-freezing in liquid nitrogen. The red trace is a simulation using an $S = 1/2$ spin system and a \mathbf{g} tensor of [2.093, 2.050, 2.003]. (B) X-band (9.37 GHz, 10 K) CW EPR spectra of *TmHydE* incubated with 20 equivalents of DTH and 10 equivalents of SAM. The spectrum shows only the SAM-bound $[4\text{Fe}-4\text{S}]^+_{\text{RS}}$ cluster in *TmHydE*, which has a \mathbf{g} tensor of [2.019, 1.882, 1.840]. (C) Depiction of the low-spin Fe(II) organometallic units supplied by syn-B and syn-B-cysteamine complexes.

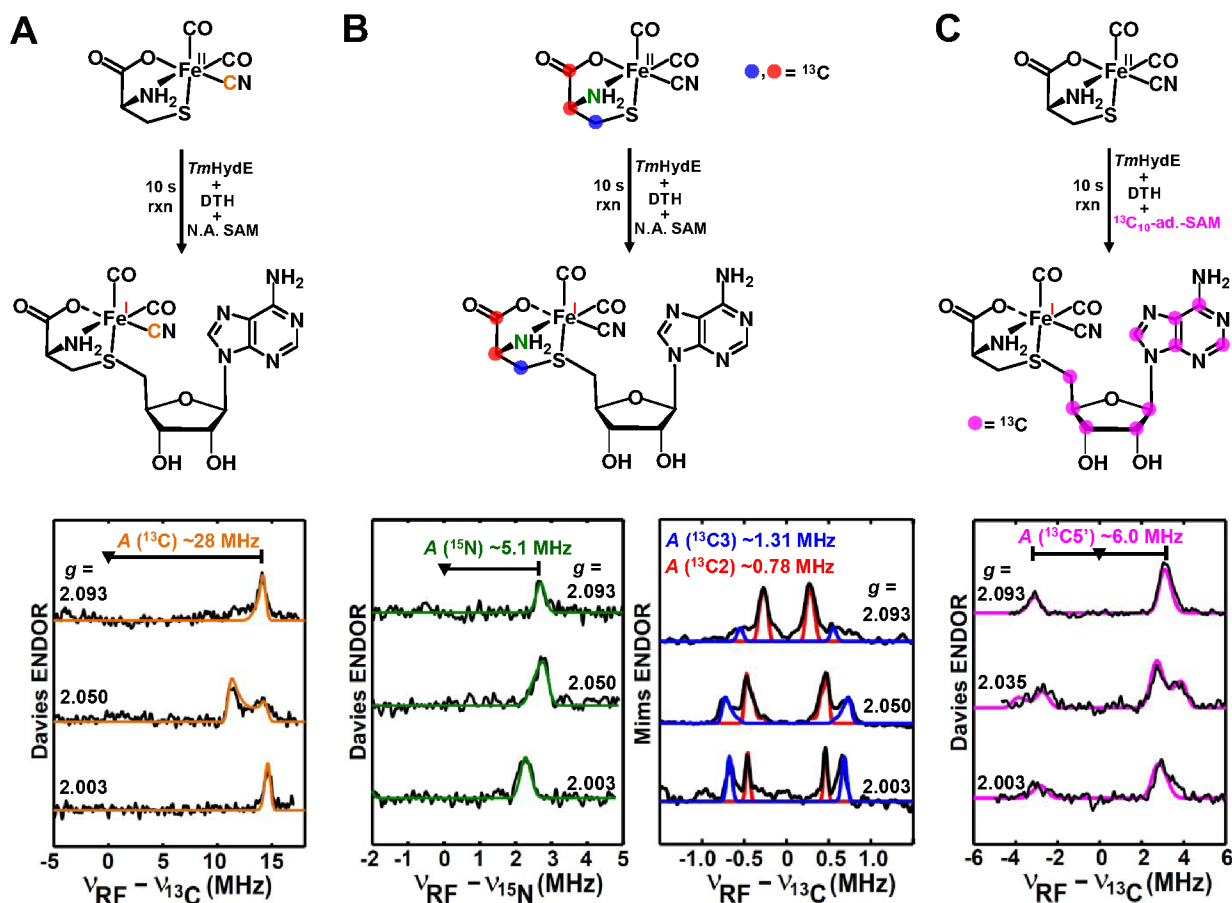


Fig. 3 (double column). ENDOR spectra (15 K) and a proposed ENDOR-based structure of the 10 s-intermediate of *TmHydE* reaction, using (A) syn-B- ^{13}CN , (B) syn-B- $^{13}\text{C}_3/^{15}\text{N}$ -Cys or (C) $^{13}\text{C}_{10}$ -adenosine-labeled SAM. The ^{13}C hyperfine-coupling signals of ^{13}CN (A) and $^{13}\text{C}5'$ (C), as well as the ^{15}N hyperfine-coupling signal (B, left panel) from $^{13}\text{C}_3/^{15}\text{N}$ -Cys were detected by Q-band Davies-ENDOR with experimental parameters of: 34.06 GHz, microwave inversion pulse $\pi = 80$ ns, $\pi/2 = 12$ ns, $\tau = 300$ ns, and RF pulse = 20 μs . The two sets of ^{13}C hyperfine-coupling signals from $^{13}\text{C}_3/^{15}\text{N}$ -Cys (B, right panel) were detected by Q-band Mims-ENDOR with experimental parameters as: 34.04 GHz, $\pi/2 = 12$ ns, $\tau = 300$ ns, and RF pulse = 20 μs . All the experimental spectra are in black, and the simulated spectrum are in color representing the labeled isotopes. The simulation parameters are given in SI.

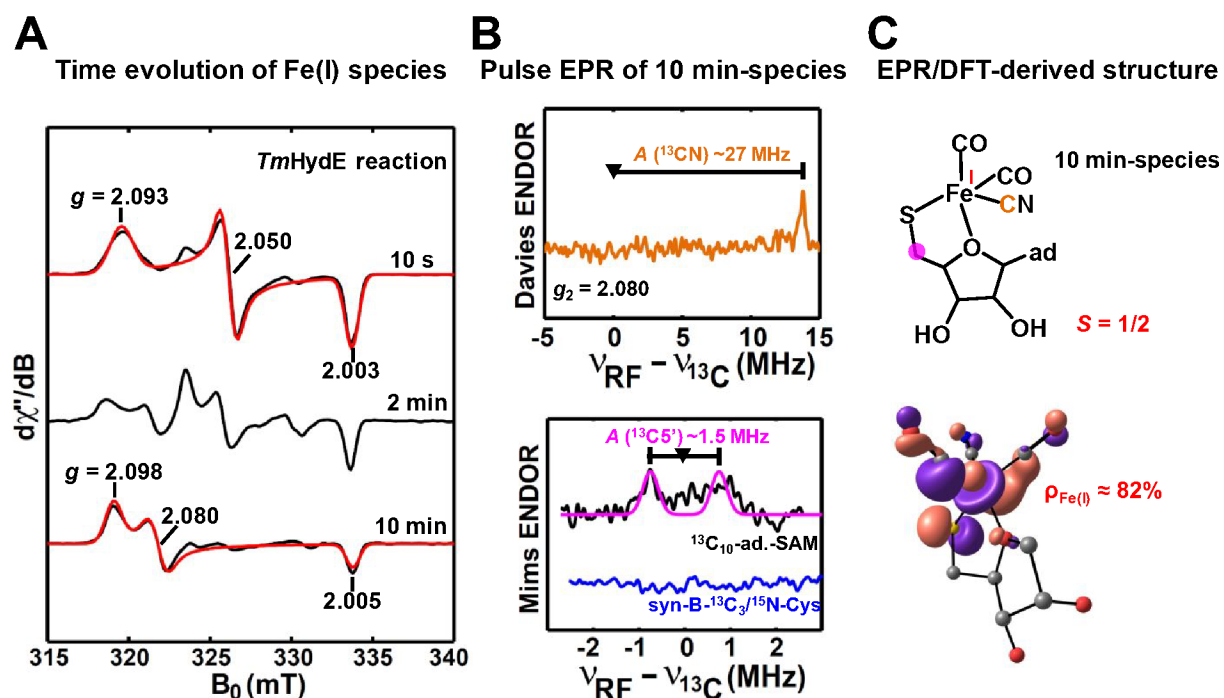


Fig. 4 (double column). (A) X-band (9.37 GHz, 35 K) CW EPR spectra (black) of *TmHydE* reaction mixtures freeze quenched at 10 s, 2 min, or 10 min. The top red trace is the simulation using an $S = 1/2$ spin system and a g tensor of [2.093, 2.050, 2.003]. The bottom red trace is the simulation using an $S = 1/2$ spin system and a g tensor of [2.098, 2.080, 2.005]. The analysis of the more complicated 2 min-spectrum is provided in Fig. S7. (B) Q-band Davies- and Mims-ENDOR spectra of the isotopically-labeled 10 min-species of *TmHydE* reaction by syn-B- ^{13}CN (orange trace), syn-B- $^{13}C_3/^{15}N$ -Cys (blue trace) or $^{13}C_{10}$ -adenosine-labeled SAM (experimental spectrum in black and the simulated spectrum in magenta). (C) The proposed structure of the 10 min-species based on EPR/ENDOR data. DFT calculated SOMO (singly occupied molecular orbital) of the 10 min-species is shown with isosurface value 0.05 a.u. (see Fig. S8 for details)

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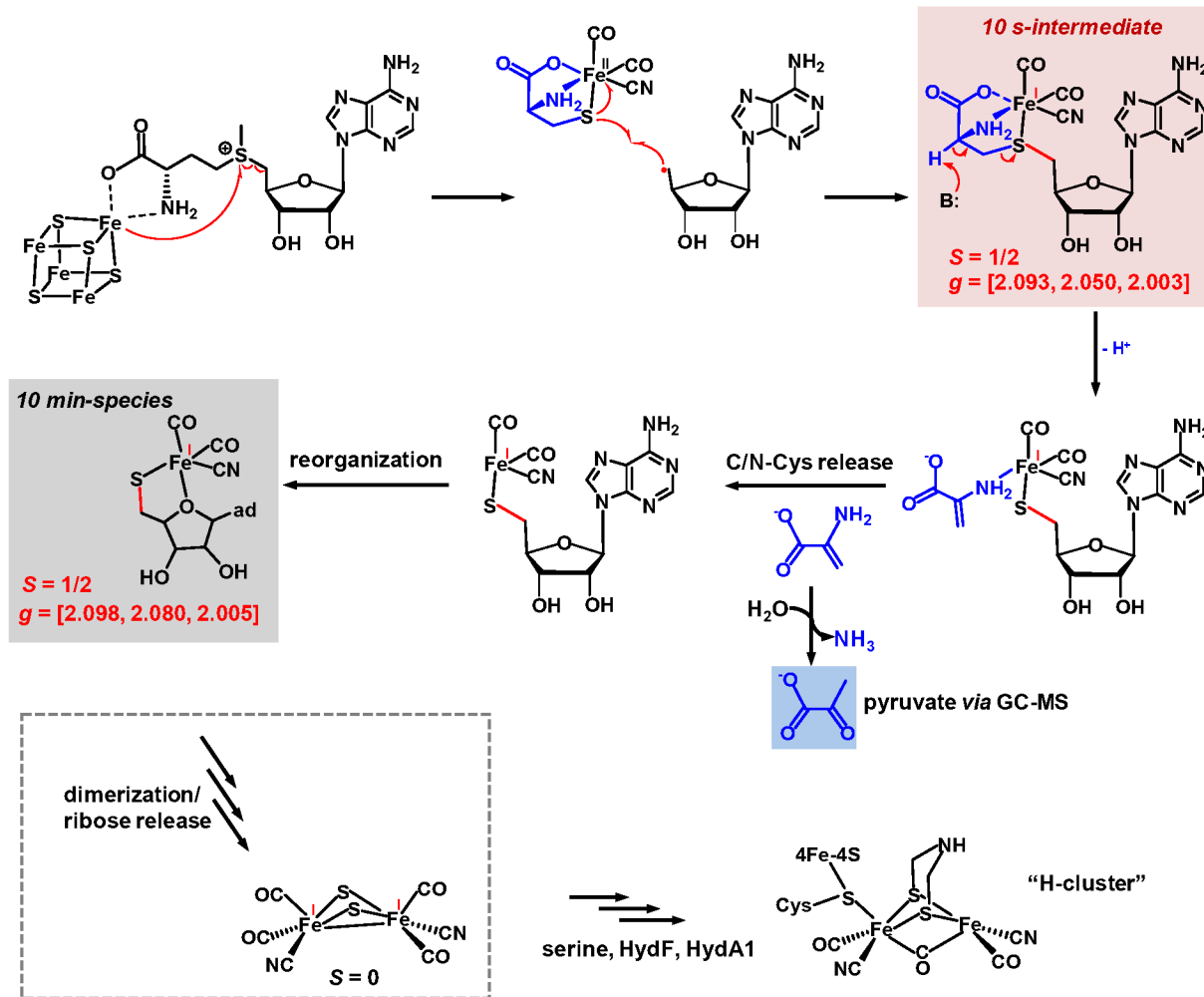


Fig. 5 (double column) Overview of our proposed reaction pathway of HydE reacting with the [Fe^{II}(Cys)(CO)₂(CN)] synthon. The Fe(I)Fe(I) dimer species shown following the 10 min-species (within the dashed box) is currently speculative.

TOC:

