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Semisynthetic Maturation of [FeFe]-Hydrogenase using $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$: Key Roles for HydF and GTP

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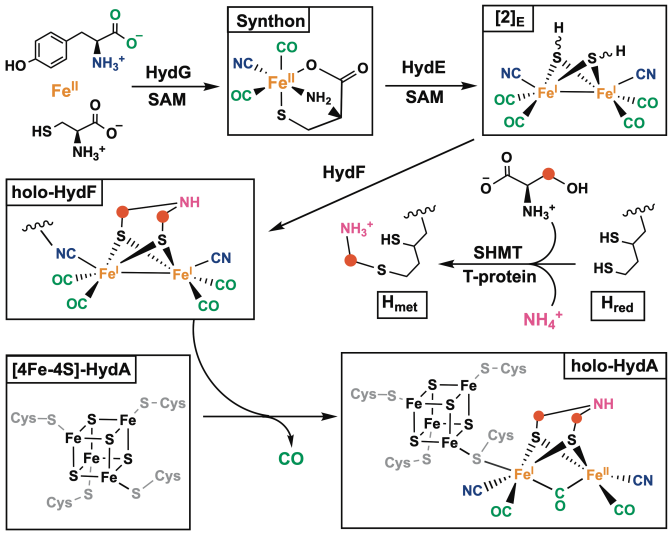
Here we describe maturation of the [FeFe]-hydrogenase from its [4Fe-4S]-bound precursor state by using the synthetic complex $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$ together with HydF and components of the glycine cleavage system, but in the absence of the maturases HydE and HydG. This semisynthetic and fully-defined maturation provides new insights into the nature of H-cluster biosynthesis.

The organometallic H-cluster at the active site of [FeFe]-hydrogenase (HydA) consists of a [4Fe-4S] cubane bridged via a cysteine to a 2Fe subcluster ($[2Fe]_H$) having CO, CN^-, and dithiomethylamine (DTMA) ligands.[1-3] The H-cluster is essential to catalysis, with reversible H^+ reduction to H_2 occuring at the distal iron site of the $[2Fe]_H$ subcluster. The [4Fe-4S] subcluster of the H-cluster is assembled on HydA by general iron-sulfur cluster assembly machinery,[4] while the [2Fe] subcluster is built and installed on HydA in a separate process referred to as hydrogenase maturation (Scheme 1).[5-8] The synthesis of the $[2Fe]_H$ subcluster is accomplished in part by the dedicated maturation enzymes HydE, HydF, and HydG, which were first shown to be essential to this process by Posewitz, King, and coworkers.[9-11] In subsequent *in vitro* experiments, active hydrogenase was generated by incubating HydA with HydE, HydF, and HydG together with S-adenosyl-L-methionine (SAM), tyrosine, guanosine 5'-triphosphate (GTP), and other small molecules, and with an absolute requirement for the presence of clarified *E. coli* cell lysate.[12, 13]

HydF is a cation-activated GTPase that binds iron-sulfur clusters, and has been implicated as a scaffold for assembly of the [2Fe] subcluster.[14-17] HydG is a radical SAM enzyme that catalyzes the radical decomposition of tyrosine to produce p-cresol, CO, and CN^-.[18-23] The diatomic ligands bind to a dangler iron appended to an auxiliary [4Fe-4S] cluster of HydG, ultimately forming a $[Fe^{II}(CO)_2(CN)Cys]^{2-}$ synthon (Scheme 1).[24-28] The synthon is a substrate for a second radical SAM maturase HydE, with the HydE-catalyzed reaction putatively forming a $[(Fe^{I})_2(\mu\text{-}SH)_2(CO)_4(CN)_2]^{2-}$ dinuclear product that is transferred to HydF (**Scheme 1**).[29, 30]

A semisynthetic approach described by Britt and Rauchfuss showed that HydA can be matured to an active enzyme in the absence of HydE and HydG by using clarified lysates of cells expressing HydF and HydA, together with a synthetic [2Fe] subcluster precursor $[K_2(18\text{-crown-6})_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$, demonstrating that the radical SAM maturases are not necessary for synthesis of the DTMA ligand of the H-cluster, and implicating a role for HydF in the synthesis of this species.[31] This work required the presence of cell lysate, as did prior *in vitro* maturations, which raised a key question: what are the unknown components provided by lysate essential for HydA



Scheme 1 Maturation of the [FeFe]-hydrogenase.

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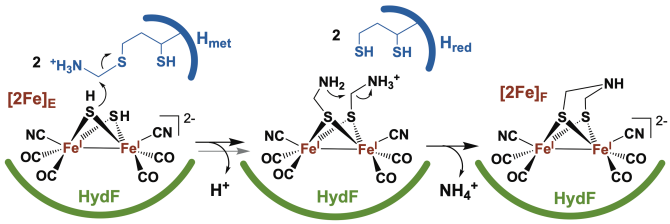
maturation? In a major recent advance, the unknown lysate components were identified as proteins and substrates of the glycine cleavage system (GCS), enabling development of a lysate-free defined maturation system consisting of the maturases HydE, HydF, and HydG, together with GCS components H-protein, T-protein, serine hydroxymethyltransferase (SHMT), serine, and ammonium.[32] This work demonstrated an essential role for the aminomethyl-lipoyl-H-protein (H_{met}) in the biosynthesis of the DTMA ligand of the H-cluster,[32] confirming serine as the source of the DTMA carbons (as previously shown)[33] and establishing ammonium as the ultimate source of the DTMA nitrogen (**Scheme I**).[32]

We have hypothesized that H_{met} interacts with the putative dinuclear product of HydE ($[(Fe^{I})_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$ or $[2Fe]_E$) complexed to HydF, installing the DTMA ligand to yield the $[2Fe]_F$ ($[(Fe^{I})_2(DTMA)(CN)_2(CO)_4]^{2-}$) subcluster (**Scheme 2**).[32] Here we use biophysical and analytical approaches to demonstrate that $[2Fe]_E$ binds to HydF. Further, we use hydrogen production assays to establish that the HydF – $[2Fe]_E$ complex, combined with our GCS-based defined maturation system (in the absence of HydE and HydG), generates a mature, active [FeFe]-hydrogenase.



Scheme 2. Proposed biosynthesis of DTMA on $[2Fe]_E$.

The synthetic [2Fe] subcluster precursor $[K_2(18\text{-crown-}6)_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$ (a source of $[(Fe^{I})_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$ or $[2Fe]_E$) was synthesized according to literature precedent (see ESI).[31] The crystalline compound was dissolved in buffer and then a 7.3-fold excess was added to purified HydF under anaerobic conditions in the dark. Size exclusion chromatography showed increased absorbance of the protein peak consistent with binding and co-elution of $[2Fe]_E$ with HydF (**Fig. S1**). When purified HydF (3.2 ± 0.3 Fe/monomer) was incubated with excess $[2Fe]_E$ for 15 – 20 min in the dark, and then subjected to buffer exchange using either Amicon spin filter devices or gel filtration, the resulting protein exhibited increased iron numbers ranging from 4.5 ± 0.1 to 5.1 ± 0.2 Fe/monomer. These increases of 1.3 to 2 Fe/monomer are consistent with the binding of $[2Fe]_E$ to HydF, which would give an increase of 2 Fe/monomer for the ideal situation where one $[2Fe]_E$ binds to each HydF monomer. UV-visible spectroscopy revealed the presence of an absorption band at 350 nm in these $[2Fe]_E$/HydF samples that is not present in HydF alone; this 350 nm absorption feature is characteristic of $[2Fe]_E$ (**Fig. 1 and Fig. S2**). EPR spectroscopy of HydF in the presence of excess $[2Fe]_E$ shows subtle *g*-value pertubations of the $[4Fe\text{-}4S]^{1+}$ cluster (*g* = 2.062, 1.879, 1.858) relative to HydF alone (*g* = 2.058, 1.879, 1.862), however the predominant effect is an increase in the

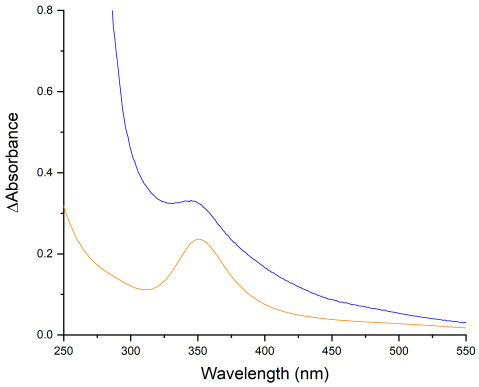


Fig. 1 UV-Vis evidence for $[2Fe]_E$ binding to HydF. Blue, difference spectrum of HydF incubated with excess $[2Fe]_E$ and then buffer exchanged, minus the starting HydF. During this treatment the HydF iron content increases from 3.2 to 4.5, and a feature at 350 nm appears. Orange, free $[2Fe]_E$ in 50 mM HEPES pH 7.5 buffer at a concentration of ~28 μM; the concentration correlates to amount of $[2Fe]_E$ expected to be bound to HydF based on the difference in iron number.

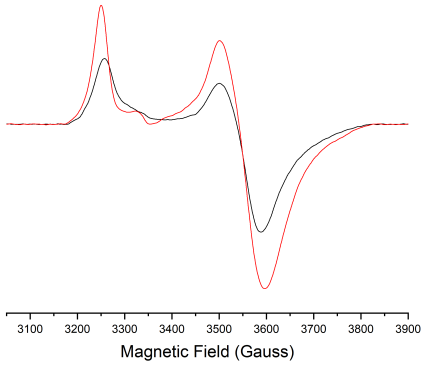


Fig. 2 EPR spectra of reduced HydF in the absence (black) and presence of $[2Fe]_E$ (red). HydF (40 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) was reduced with 2 mM DTT and 2 mM DT for 10 min before being flash frozen (black). HydF (40 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) was reduced with 2 mM DTT and 2 mM DT, and then a 7.3-fold excess of $[2Fe]_E$ was added and the sample was flash frozen. EPR parameters: 12.0 K, 1.0 mW power, 4 scans averaged.

intensity of the EPR signal (**Fig. 2 and Fig. S3–S5**). Photolysis of the gel-filtered HydF-$[2Fe]_E$ complex with a xenon arc lamp resulted in the production of CO, which was detected by binding to the CO trapping agent H64L myoglobin (Mb) (see ESI), the latter of which we have previously used to measure CO production by HydG.[23, 34] The production of the CO-bound H64L Mb upon photolysis of $[2Fe]_E$/HydF is indicated by the distinct shift in the heme Soret band (**Fig. S6**), demonstrating that CO-containing $[2Fe]_E$ is bound to HydF. Together, the above results support a model in which the synthetic $[2Fe]_E$ binds to HydF prior to DTMA ligand installation.

In order to test our hypothesis that H_{met} interacts with $[2Fe]_E$ bound to HydF to install the DTMA ligand to yield the $[2Fe]_F$ subcluster (**Scheme 2**), we carried out HydA maturation experiments with HydF, $[2Fe]_E$, HydA, and the H_{met} generation system previously described.[32] This system consists of SHMT, T-protein, serine, and NH_4Cl, as well as several other small molecule components including GTP (see ESI).[32] These semisynthetic defined maturation reactions provided [FeFe]-hydrogenase activities of 95 µmol/min/mg (**Fig. 3**), a number comparable to recent reports for semisynthetic maturation using cell lysate rather than the defined components described here.[31] These results demonstrate that components of the GCS can replace cell lysate to support maturation using the synthetic $[2Fe]_E$ H-cluster precursor, and support the hypothesis that H_{met} interacts with the HydF-$[2Fe]_E$ complex to synthesize the DTMA ligand.

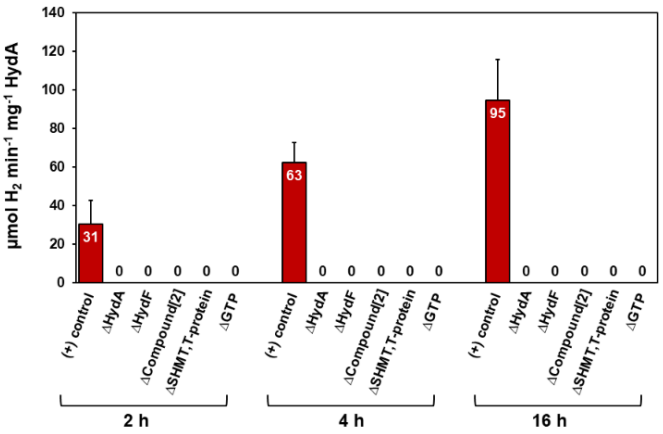


Fig. 3 Maturation of the [FeFe]-hydrogenase with a semisynthetic defined system. The full assay contains HydF, HydA, $[2Fe]_E$, SHMT, T-protein, serine, NH_4Cl, $FeSO_4$, cysteine, GTP, PLP, DTT, and dithionite, but no HydE or HydG. Omitting key components, as shown, results in no maturation.

Maturation of HydA in this semisynthetic approach is absolutely dependent on the presence of synthetic $[2Fe]_E$ (**Fig. 3**), supporting the idea that $[2Fe]_E$ functionally bypasses the radical SAM HydE and HydG maturases and that $[2Fe]_E$ is the product of HydE. HydF is also essential to maturation with synthetic $[2Fe]_E$, consistent with the proposal that $[2Fe]_E$ must bind to HydF prior to synthesis of the DTMA ligand, and that neither $[2Fe]_E$ free in solution nor bound to HydA is a suitable substrate for DTMA ligand biosynthesis. Furthermore, studies looking at the dependence of maturation on the concentration of $[2Fe]_E$ reveal that excess $[2Fe]_E$ beyond the concentration of HydF does not improve maturation (**Fig. S7**), indicating that the 1:1 $[2Fe]_E$/HydF complex is the relevant species in maturation. HydA was also found to be absolutely required for H_2 production, indicating that neither free $[2Fe]_E$ nor $[2Fe]_F$ bound to HydF give rise to background activity (**Fig. 3**). Hydrogen production is also not observed in these assays if the GCS components T-protein (which includes co-purified H-protein)[32] and SHMT are omitted, indicating these proteins are essential to convert HydF-bound $[2Fe]_E$ to a DTMA-bound $[2Fe]_F$.

An intriguing result of the HydA maturation assays shown in Figure 3 is that no H_2 production activity was observed unless GTP was present. HydF is a GTPase, however the role of GTP hydrolysis during [FeFe]-hydrogenase maturation is not well understood.[17, 35] Prior work demonstrated that HydF GTPase activity is not required for the transfer of $[2Fe]_F$ to apo-HydA,[17] which led to the proposal that GTP hydrolysis was involved in modulating the interactions of HydF with the other maturase enzymes, HydE and/or HydG.[17, 35, 36] However in the HydA maturation experiments presented here, HydE and HydG are not present, and yet GTP is still absolutely required for maturation. These results indicate that HydF-GTPase activity is associated with the interaction of HydF with H_{met} or the H_{met}/T-protein complex to facilitate DTMA ligand biosynthesis on HydF bound $[2Fe]_E$, consistent with the proposed biosynthesis of the DTMA ligand shown in Scheme 2. The details of these protein-protein interactions, and the specific role for GTP binding and hydrolysis, await further studies.

The synthetic complex $[2Fe]_F$ was shown to bind to HydF via a CN^- bridge to the [4Fe-4S] cluster of HydF, and this $[2Fe]_F$/HydF complex was able to mature apo-HydA to an active hydrogenase.[37] Activation-competent HydF produced biosynthetically by co-expression with HydE and HydG in *E. coli* also harbors a 2Fe subcluster with spectroscopic properties similar to the $[2Fe]_F$/HydF semisynthetic complex.[38] Thus one might expect that $[2Fe]_E$ binds to HydF via a bridging CN^- to the [4Fe-4S] cluster, similar to $[2Fe]_F$/HydF. However, a recent report showed that synthetic $[2Fe]_F$ can bind to HydF and activate HydA even in the absence of the [4Fe-4S] cluster.[39] Further studies are needed to determine the details of how $[2Fe]_E$ binds to HydF, and specifically whether $[2Fe]_E$ binds directly to the [4Fe-4S] cluster of HydF.

Here we demonstrate that the [FeFe]-hydrogenase can be matured in the absence of HydE and HydG using a fully-defined semisynthetic system. The synthetic complex $[2Fe]_E$ is shown to bind to HydF, based on increasing iron numbers, characteristic EPR and UV-Vis absorption changes, and the ability to detect CO liberated via photolysis of $[2Fe]_E$ bound to HydF. The HydF-$[2Fe]_E$ complex is required for the subsequent maturation step in which H_{met} or an H_{met}/T-protein complex interacts with $[2Fe]_E$-HydF to install the DTMA ligand (**Scheme 2**), in a process that is dependent on the presence of GTP.

Author contributions are as follows: B. Balci, investigation, methodology, editing; R. D. O'Neill, investigation; E. M. Shepard, investigation, methodology, editing; A. Pagnier, investigation, editing; A. Marlott, investigation; M. T. Mock, supervision, methodology, writing, editing; W. E. Broderick, conceptualization, methodology, supervision, writing, editing; J. B. Broderick, conceptualization, methodology, supervision, writing, editing, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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