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[FeFe]-Hydrogenase: Defined Lysate-Free Maturation Reveals a Key Role for Lipoyl-H-Protein in DTMA Ligand Biosynthesis

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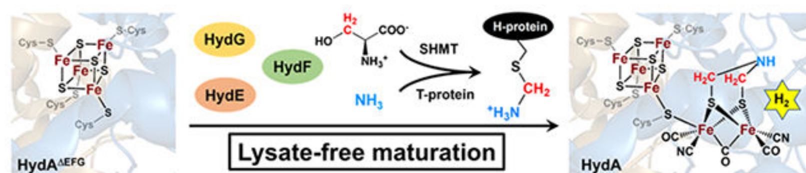
Abstract

Maturation of [FeFe]-hydrogenase (HydA) involves synthesis of a CO, CN^-, and dithiomethylamine (DTMA)-coordinated 2Fe subcluster that is inserted into HydA to make the active hydrogenase. This process requires three maturation enzymes: the radical S-adenosyl-L-methionine (SAM) enzymes HydE and HydG, and the GTPase HydF. *In vitro* maturation with purified maturation enzymes has been possible only when clarified cell lysate was added, with the lysate presumably providing essential components for DTMA synthesis and delivery. Here we report maturation of [FeFe]-hydrogenase using a fully defined system that includes components of the glycine cleavage system (GCS), but no cell lysate. Our results reveal for the first time an essential role for the aminomethyl-lipoyl-H-protein of the GCS in hydrogenase maturation and the synthesis of the DTMA ligand of the H-cluster. In addition, we show that ammonia is the source of the bridgehead nitrogen of DTMA.

Graphical Abstract



The aminomethyl-lipoyl-H-protein (H_{met}) of the glycine cleavage system (GCS) is shown to play a key role in maturation of the [FeFe]-hydrogenase. Including H_{met}, or H_{met} plus other components of the GCS, allows maturation by purified HydE, HydF, and HydG in the absence of cell lysate for the first time.

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Keywords

hydrogenase maturation; lipoyl-H-protein; biosynthesis; glycine cleavage system; dithiomethylamine

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[FeFe]-hydrogenase (HydA) catalyzes the reversible reduction of protons to H_2 at a complex metal cluster, the H-cluster, composed of a [4Fe-4S] cluster bridged via a cysteine thiolate to a [2Fe] subcluster coordinated by CO, CN^- and dithiomethylamine (DTMA) ligands (Figure 1).[1] [FeFe]-hydrogenases can catalyze rates of H_2 production that are approximately 100x higher than other hydrogenases, making HydA an appealing target for biohydrogen production technologies.[2] Early efforts to heterologously express [FeFe]-hydrogenase, however, led to inactive enzyme, a major obstacle to practical applications of this enzyme.[3] Subsequent studies showed that three maturation enzymes, HydE, HydF, and HydG, were required to produce an active [FeFe]-hydrogenase.[4]

HydF is a GTPase that binds redox-active iron-sulfur clusters and serves as a scaffold for assembly of a 2Fe precursor ($[2Fe]_F$) delivered to HydA to yield active hydrogenase.[5] HydF can be loaded with $[2Fe]_F$ enzymatically by co-expression with HydE and HydG, yielding a $[2Fe]_F$ subcluster resembling the [2Fe] subcluster of the H-cluster ($[2Fe]_H$).[6] In an alternative semi-synthetic approach, pre-formed synthetic [2Fe] clusters coordinated by CO, CN^-, and DTMA can be used to load HydF, which then can activate HydA.[7] HydG synthesizes the CO and CN^- ligands from tyrosine,[8] and also provides iron in the form of a $[Fe(Cys)(CO)_2(CN)]$ synthon.[9] HydE binds a synthetic analog of this synthon, and uses it as a substrate to catalyze an adenosylation reaction,[10] and may convert two of these adenosylated Fe(I) synthon units to a [2Fe] subcluster precursor.[11] The origin of the DTMA ligand is not well understood. It was thought to arise from HydE catalysis[12] but now appears to be formed independent of HydE.[11] Maturation of HydA ^{EFG} (HydA expressed in the absence of the maturase enzymes) can be achieved *in vitro* by combining extracts of *E. coli* cells overexpressing the individual maturases, or by using purified maturase enzymes together with *E. coli* cell lysate.[8d, 13] In work to date, the requirement for the *E. coli* lysate (20% to 100% v/v) has been absolute.[4c, 4d, 8d, 9a, 11, 13–14]

We report here development of a fully defined enzymatic system for maturation of HydA using HydE, HydF, and HydG, their substrates and reductants, and components of the glycine cleavage system (GCS, Scheme S1),[15] but without *E. coli* lysate. The results reveal a key role for the aminomethyl-lipoyl-H-protein (H_{met}) as a precursor of the DTMA ligand of the H-cluster.

To better understand the chemistry of H-cluster maturation, we pursued fractionation of *E. coli* cell lysate to identify component(s) responsible for its requirement during maturation. These efforts revealed one or more components of a low-molecular weight (LMW) fraction (10 – 15 kDa, Figure S1) of lysate could also support maturation. Essential component(s) from *E. coli* lysate were inactivated by heat/acid treatment, suggesting they may be proteins (Figure S2). Shotgun proteomics revealed the presence of carrier proteins including the acyl-carrier protein (ACP) in the LMW fraction, suggesting the involvement of a small carrier protein in hydrogenase maturation, perhaps in the still poorly understood synthesis

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of the DTMA ligand, as suggested by Juan Fontecilla-Camps (personal communication). However, no detectable HydA activity was observed following *in vitro* HydA maturation with holo- or lipoyl-ACP in place of *E. coli* cell lysate, and with purified HydE, HydF, HydG, SAM, and other small molecule components (see SI).

We next considered the H-protein of the GCS (Scheme S1), as a potential key component of HydA maturation. Lipoylated H-protein acts as a carrier between protein components of the GCS (Scheme S1), and can be in the disulfide form (H_{ox}), the reduced form (H_{red}), or the aminomethyl form (H_{met}). Maturation reactions carried out with H_{red} in the absence of cell lysate provided no detectable hydrogenase activity (Figure 2). H_{met} was synthesized enzymatically from H_{ox} using the P-protein, PLP, and glycine (Scheme S1), purified, and verified via mass spectrometry (Figure S3). HydA maturation with H_{met} added to our defined system resulted in active HydA, as evidenced by H_2 gas production (Figure 2). Increasing the amount of H_{met} provided a significant increase in H_2 (Figure 2), demonstrating that HydA activation is dependent on H_{met}. To our knowledge, this is the first report of biological HydA ^{EFG} conversion to active hydrogenase in the absence of cell lysate, and points to H_{met} as a previously unrecognized essential component.

In these experiments, the level of activation of HydA ^{EFG} in the presence of H_{met} is nonetheless low, requiring 24 h incubation to detect small amounts of H_2. The low hydrogenase activity may be due to instability of H_{met}, which is known to readily hydrolyze, especially in the presence of partner proteins.[16] We reasoned that if this is so, regeneration of H_{met} *in situ* would improve the hydrogenase maturation. Regeneration of H_{met} from H_{red} *in situ* under the reducing conditions required for maturation was accomplished by including aminomethyltransferase (T-protein)**,** serine hydroxymethyltransferase (SHMT), serine, and NH_4^+ (Scheme 1) in the maturation mixture. HydA maturation under these conditions yielded dramatically improved hydrogenase activity, with further refinement as described below providing specific activities as high as 390 μmol/min/mg HydA (Figure 3); in comparison, holo-HydA is reported to have a specific activity of ~700 μmol/min/mg.[17]

To further probe the involvement of H_{met} of the GCS during HydA maturation, we examined the dependence of maturation on concentrations of the H_{met} regeneration components (Figure 3). Maturation is dependent on both serine and NH_4^+, with high concentrations required for optimal activity, consistent with the equilibria outlined in Scheme 1. Glycine has an inverse effect on maturation (Figure 3), consistent with the role of SHMT in producing methylene-tetrahydrofolate (CH_2-THF, Scheme 1). The maturation observed without added NH_4^+ is attributed to co-purification of NH_4^+ with T-protein, consistent with the well-defined NH_4^+ binding site in this protein.[18] THF also impacts maturation, increasing activity at low concentrations followed by a rapid decrease with increasing THF concentration (Figures 3, S5), consistent with THF involvement in both SHMT and T-protein equilibria; the dominant effect of THF is on the T-protein equilibrium, driving it towards H_{red} and away from H_{met} (Scheme 1). Maturation works without added THF because THF co-purifies with the T-protein. The dependence of HydA maturation on these components required for generation of H_{met} *in situ* further support a key role for H_{met} in HydA maturation; these components were provided by lysate in prior lysate-dependent maturations.[4c, 4d, 8d, 9a, 13b, 13c, 14]

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Our results suggest that the GCS, and specifically H_{met}, provides a precursor for DTMA biosynthesis. We propose that the aminomethyl group of H_{met} is the source of the C and N of the C-N-C framework of DTMA. Given that the aminomethyl of H_{met} is derived from the β-C of serine and NH_4^+ (Scheme 1), we set out to probe incorporation of isotopic labels from these sources into the DTMA of matured HydA. Maturation reactions were carried out in the absence of lysate (see SI), with [$^{13}C_3$,^{15}N]-serine or $^{15}NH_4Cl$ included in place of the unlabeled component. HydA was purified from the maturation mixture and oxidized with thionin to give an electron paramagnetic resonance (EPR) spectrum typical of the H_{ox} state of holo-HydA (Figure S6). Electron nuclear double resonance (ENDOR) spectroscopy was used to look for incorporation of isotopic labels into the DTMA of the H-cluster. For the [$^{13}C_3$,^{15}N]-serine samples, the spectrum collected at ~ g_1 shows two distinct ^{13}C hyperfine signals (Figure 4, red) associated with the two carbons of the DTMA ligand, as reported previously for HydA matured in the presence of lysate.[14b] The presence of signals with different hyperfine splittings for the two DTMA carbons is consistent with the prior work demonstrating their origins from serine and asymmetry in the DTMA ligand environment in holo-HydA.[14b, 19] However, this sample prepared without lysate shows no ^{15}N ENDOR response, indicating that ^{15}N from [$^{13}C_3$,^{15}N]-serine is not present in the DTMA of HydA matured lysate-free (Figure 4, black).

In our defined maturation, the T-protein uses NH_4^+ to provide the N for the aminomethyl group of H_{met} (Scheme 1), and thus NH_4^+ should be the source of the N of DTMA. ^{15}N ENDOR spectra of HydA matured in the presence of $^{15}NH_4Cl$ reveals this to be true, as the corresponding ^{15}N coupling of 1.5 MHz is observed (Figure 4, blue). Prior work showed that both N and C of DTMA could be derived from serine, but this was in the presence of *E. coli* cell lysate (72% v/v).[14b] Given that cell lysate contains enzymes such as serine dehydratase that can liberate NH_4^+ from serine, it is likely that the observed incorporation of ^{15}N from serine in the prior work was a result of $^{15}NH_4^+$ liberation due to the presence of lysate.

We demonstrate the first maturation of HydA using a defined enzymatic system, thereby revealing an essential role for H_{met} and the GCS in building the DTMA ligand of the H-cluster. H_{met} alone, when added to lysate-free maturation components, is sufficient to provide active HydA (Figure 2), revealing the key role for H_{met} during H-cluster biosynthesis. Inclusion of an H_{met} regeneration system (T-protein, SHMT, serine, and NH_4^+) affords high levels of HydA activation, with the roles of T-protein and SHMT highlighted by the dependence of activation on the concentrations of key species (Figure 3). ^{13}C/^{15}N-ENDOR spectroscopy of HydA matured using this defined system confirms that the carbon atoms of DTMA originate from serine, while the nitrogen comes from NH_4^+ (Figure 4), consistent with our previous prediction[20] and with the known chemistry of the GCS during H_{met} formation from H_{red} (Scheme 1).

The recent report that HydA can be matured in the presence of HydF, a synthetic $[Fe_2(SH)_2(CO)_4(CN)_2]^{2-}$ complex, and cell lysate,[11] together with the results presented here, lead us to propose that H_{met} interacts with the HydF-$[Fe_2(SH)_2(CO)_4(CN)_2]^{2-}$ complex to synthesize the dimethylamine backbone of DTMA. In this model, the aminomethyl group of H_{met} is functionally equivalent to the formaldehyde and ammonia

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used to synthesize the C-N-C DTMA backbone in the $[2Fe]_H$ model reported by Li and Rauchfuss.[21] We propose that during biological maturation, each sulfur of the $[2Fe]_F$ precursor cluster $[Fe_2(SH)_2(CO)_4(CN)_2]^{2-}$ nucleophilically attacks the C of an aminomethyl group of H_{met}, resulting in transfer of two aminomethyl groups from two H_{met} to the bridging sulfides of the HydF-bound $[2Fe]_F$ precursor cluster. Condensation of the aminomethyl moieties with loss of NH_4^+ would then form DTMA (Scheme 2). In this model, the sulfurs of DTMA are derived from the precursor diiron cluster, not from the lipoyl group of Hmet; use of a seleno-lipoyl-H-protein during maturation could test this hypothesis.

Supplementary Material

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Acknowledgements

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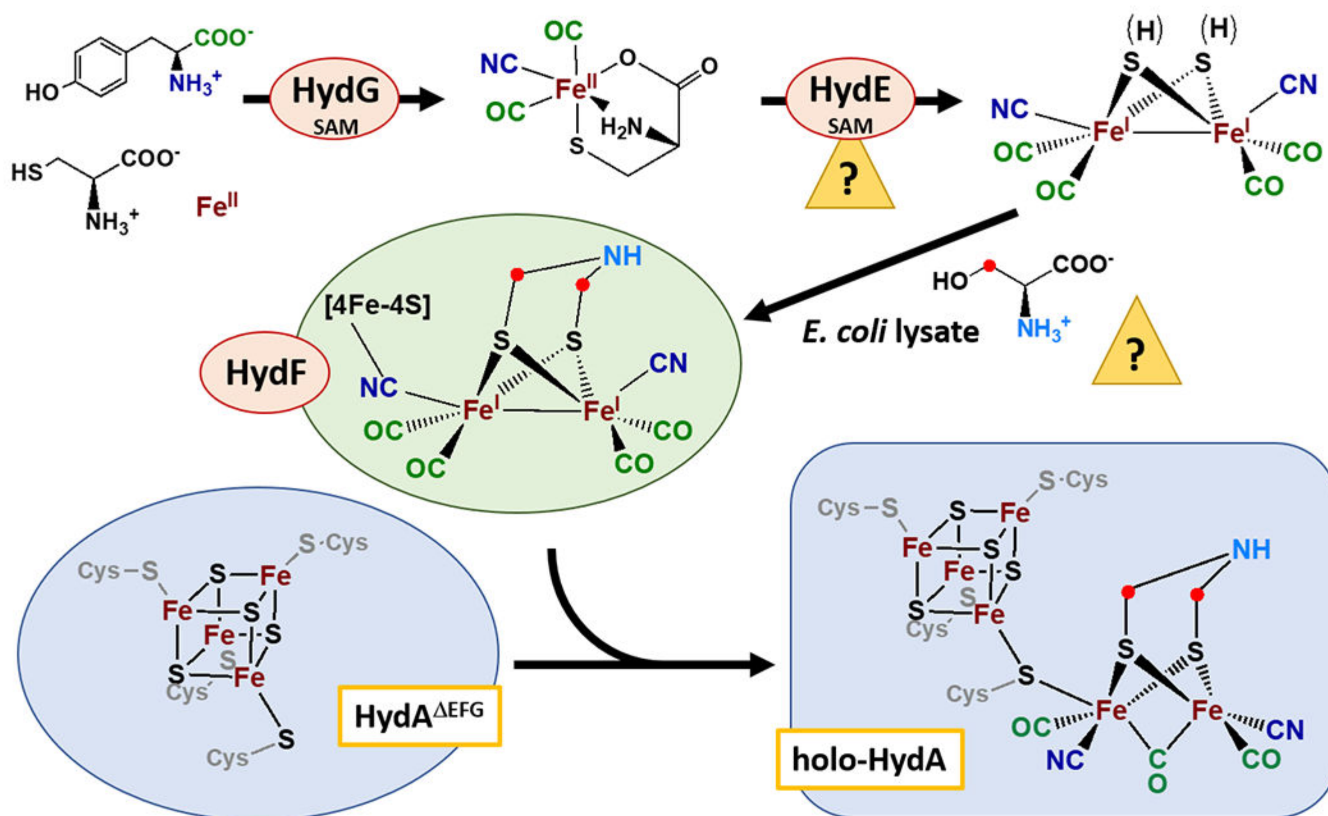

Figure 1.

Current model for biological maturation of the H-cluster of the [FeFe]-hydrogenase. Question marks indicate steps that are not fully understood.

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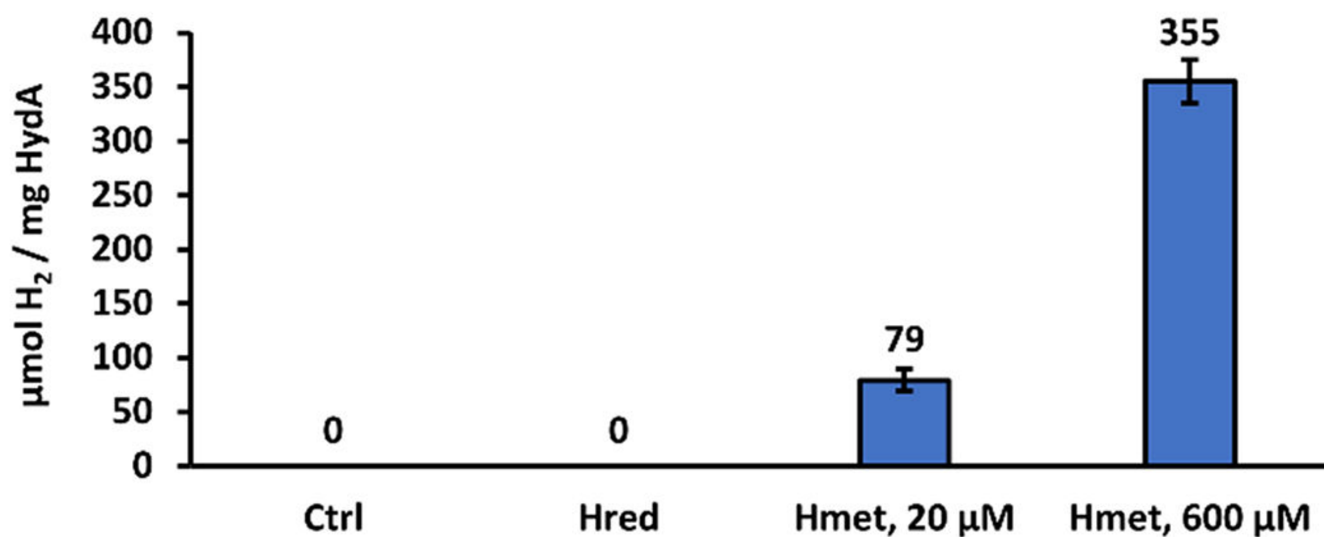

Figure 2.

Maturation of HydA without lysate, using the GCS H-protein. Without H-protein (**Ctrl**) or with lipoyl-H-protein (**H$_{red}$**) no active HydA is formed. Addition of aminomethyl-lipoyl-H-protein (**H$_{met}$**) provides maturation of HydA in a concentration-dependent manner. Assays for H_2 were run for 24 h to allow H_2 to accumulate. Conditions as reported in the SI.

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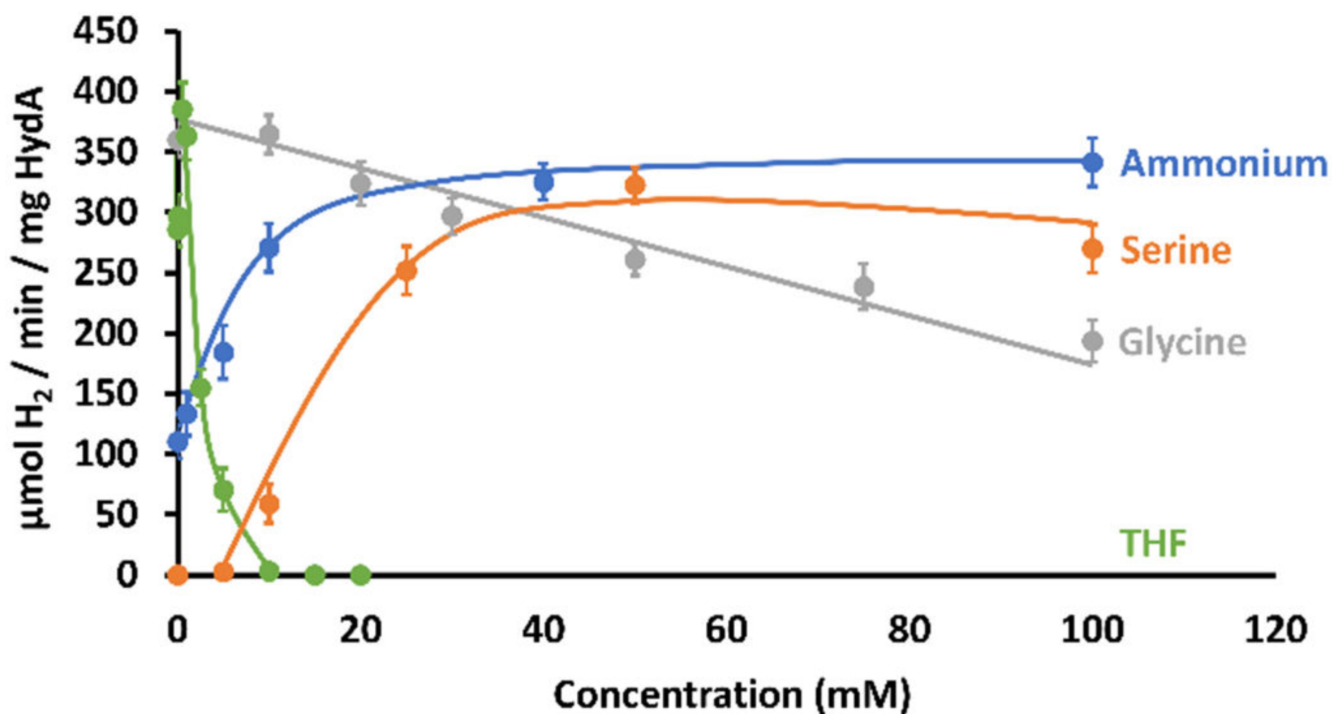

Figure 3.

Maturation of HydA in the presence of the T-protein and SHMT. Concentrations of key components were varied to test the hypothesis of the involvement of the GCS and H_{met} during hydrogenase maturation. Conditions as reported in the SI. Reactions contained no lysate.

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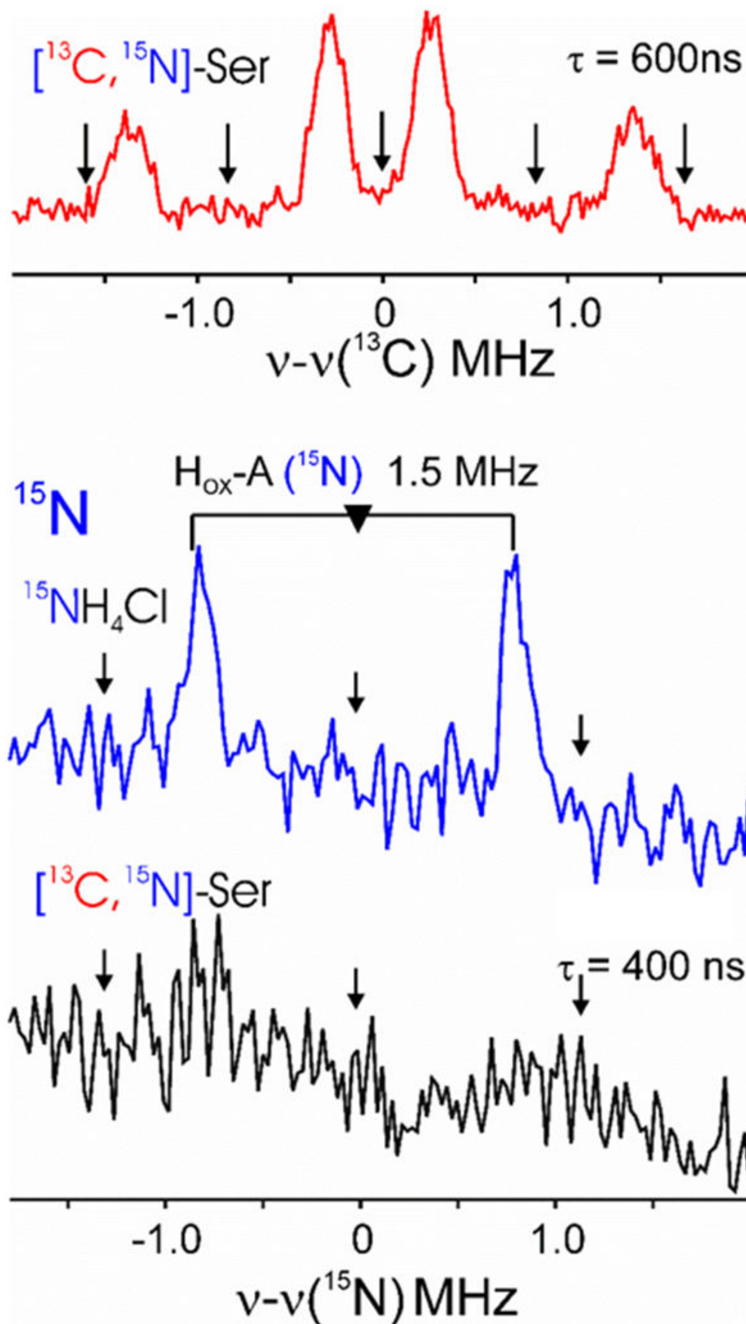

Figure 4.

35 GHz pulsed ^{13}C and ^{15}N ENDOR of HydA matured in the absence of lysate. Top, ^{13}C Mims ENDOR spectrum at g=2.093 of HydA matured with [$^{13}C_3$,^{15}N]-serine. Bottom, ^{15}N Mims ENDOR spectra at g=2.004 of HydA matured with $^{15}NH_4Cl$ (blue) and with [$^{13}C_3$,^{15}N]-serine (black). Mims holes are indicated with arrows. Microwave frequency, 34.605 GHz; rep time, 50 ms; T = 2 K. For a complete characterization of these signals, see ref 15b.

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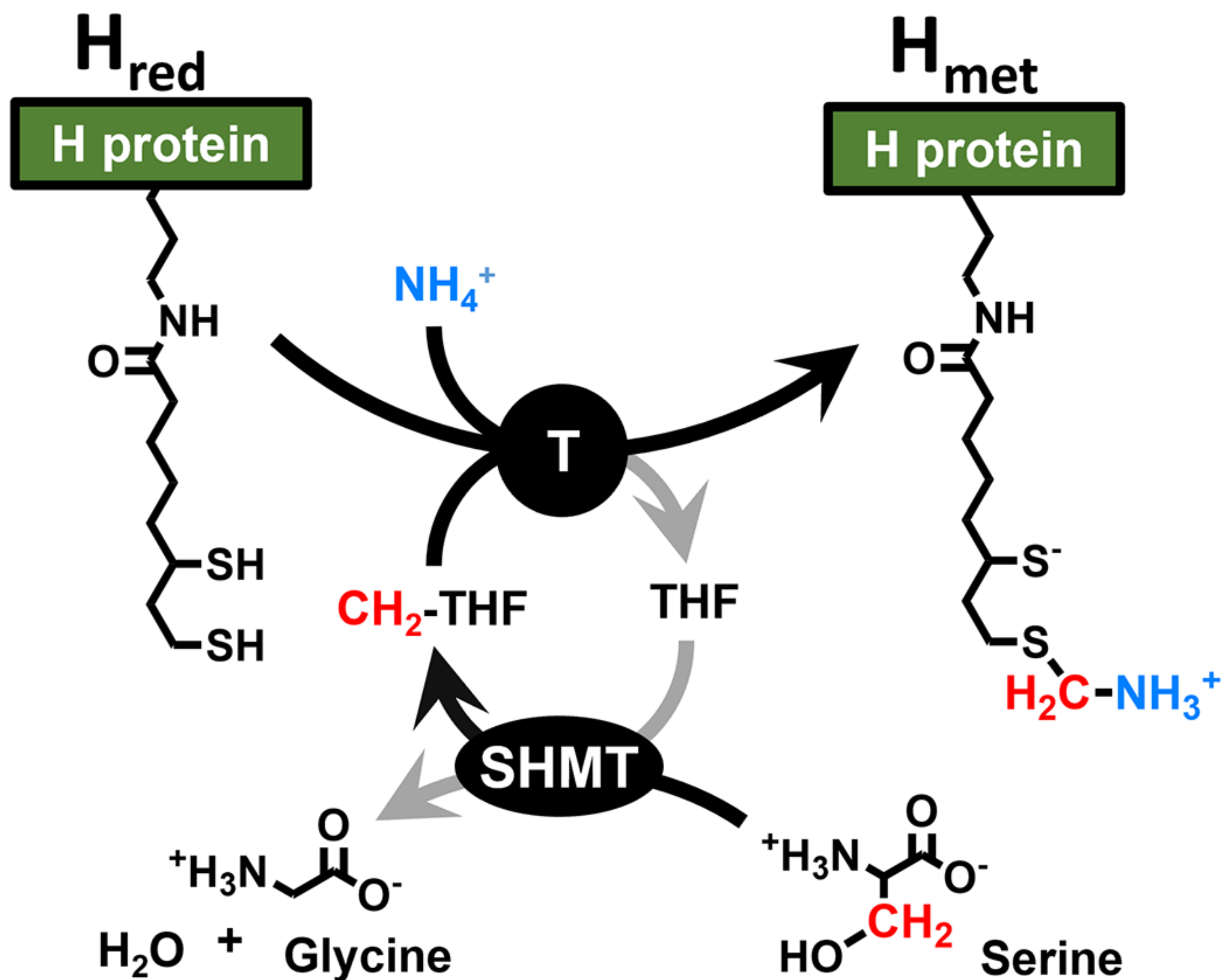

Scheme 1.

In situ generation of H_{met} from H_{red} via SHMT and the T-protein of the GCS (see Scheme S1 for the full GCS). All reactions are reversible.

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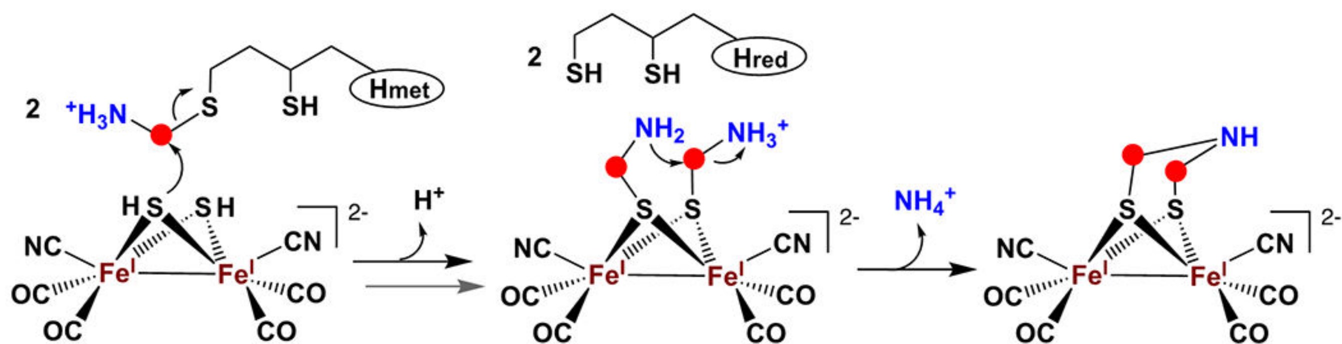

Scheme 2.

Proposed pathway for formation of the DTMA ligand of the $[2Fe]_F$ cluster on HydF.