Supporting Information

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

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Experimental Methods

E. coli Lysate Fractionation

E. coli lysate was prepared from a glycerol stock of BL21(DE3)ΔiscR:kan, which was used to inoculate 50 mL starter cultures in LB media that contained 30 μg/mL kanamycin. Large scale (3 L) growths were inoculated with 7.5 mL of the overnight culture and were subsequently incubated at 37°C for 3 hours or until $OD_{600} \approx 0.5$ was reached. The flasks were cooled on ice and centrifuged (12,000 x g, 10 min., 4˚C). The wet cell pellet was resuspended in a 1:1 ratio of anaerobic maturation buffer (100 mM HEPES, pH 8.2, 50 mM KCl) that was supplemented with PMSF (1 mM), 1% (w/v) Triton-X-100, DNase I and RNase A. The mixture was sonicated (5 min total pulse time at 60% amplitude) using a model FB505 sonic dismembrator (500 W, Fisher Scientific), followed by ultracentrifugation (100,000 x g, 1 h, 4°C) to obtain a clarified lysate, which was aliquoted and flash frozen in liquid N_2.

For lysate fractionation via gel filtration, 0.5 mL of clarified lysate was loaded onto a HiPrep 16/60 Sephacryl S-200 High Resolution column equilibrated with anaerobic maturation buffer (100 mM HEPES pH 8.2, 50 mM KCl). Eluted fractions (1.5 mL in volume) were collected and their ability to mature $HydA^{\Delta EFG}$ was assessed in our *in vitro* activation assay, as previously described.[3] To calibrate the column, 0.5 mL of gel filtration standard (Bio-Rad cat# 151-1901) containing 0.5 mg of vitamin B12 (1,350 Da), 2.5 mg of myoglobin (17,000 Da), 5.0 mg of ovalbumin (44,000 Da), 5.0 mg of γ-globulin (158,000 Da) and 5.0 mg of thyroglobulin (670,000 Da) was loaded onto the column in the same conditions than the lysate (flow rate, buffer composition). Thyroglobulin peak was excluded from the calibration curve calculation.

To test the impact of protein denaturation in the cell extract preparation, 1 mL of clarified lysate was either treated with heat or acid before being added to our *in vitro* maturation reaction as described previously.[3] The clarified lysate was incubated at 80°C for 20 min and then centrifuged to pellet the precipitated proteins. Alternatively, additions of an HCl solution (100 mM) were slowly made to the clarified lysate until protein precipitation was observed. After centrifugation, the pH of the supernatant was adjusted to ~8.

Expression and purification of *C.a.* maturases and His-tagged-*Cr*HydA

The expression, isolation and reconstitution of His-tagged *Clostridium acetobutylicum* (*C.a.*) maturases and His-tagged HydA from *Chlamydomonas reinhardtii* (*C.r.*) followed previously published protocols: *Ca*HydE[1], *Ca*HydF[2], and *Ca*HydG[3] and *Cr*HydA.[3]

StrepTagged-*Cr*HydA expression and purification

The preparation of truncated *Cr*HydA (residues 2-56 removed) with a C-terminal StrepTag was performed as described previously[4] with minor modifications. The protein sequence is as follows:

MAAPAAEAPLSHVQQALAELAKPKDDPTRKHVCVQVAPAVRVAIAETLGL
APGATTPKQLAEGLRRLGFDEVFDTLFGADLTIMEEGSELLHRLTEHLEAHP
HSDEPLPMFTSCCPGWIAMLEKSYPDLIPYVSSCKSPQMMLAAMVKSYLAE
KKGIAPKDMVMVSIMPCTRKQSEADRDWFCVDADPTLRQLDHVITTVELGN
IFKERGINLAELPEGEWDNPMGVGSGAGVLFGTTGGVMEAALRTAYELFTG
TPLPRLSLSEVRGMDGIKETNITMVPAPGSKFEELLKHRAAARAEAAAHGTP

GPLAWDGGAGFTSEDGRGGITLRVAVANGLGNAKKLITKMQAGEAKYDFV
EIMACPAGCVGGGGQPRSTDKAITQKRQAALYNLDEKSTLRRSHENPSIREL
YDTYLGEPLGHKAHELLHTHYVAGGVEEKDEKKLEIEGRQLGWSHPQFEK

The recombinant *CrHydA* gene was expressed using an *E. coli* BL21 strain containing the petDuet-1:CrHydA1,hydE plasmid. After inoculation from overnight starter cultures, cell culture was carried out in phosphate buffered LB media (9 L total in 6 x 2.8 L Fernbach flasks) supplemented with carbenicillin (200 µg/mL) and maintained at 37˚C with shaking (~220 rpm) to an OD_{600} of 0.8. The cultures were chilled to 16 – 20˚C, induced with IPTG (1 mM), and incubated overnight at 16 – 20˚C while shaking (~220 rpm). To harvest the cells, the cultures were centrifuged (12,000 x g, 10 min, 4˚C) and the wet cell pellets were immediately frozen in liquid nitrogen and stored at –80˚C. To isolate *Cr*HydA, all lysis and purification steps were performed in an anaerobic Coy vinyl glove box. At a ratio of ~2 mL of buffer per 1 g of cell paste, lysis was performed at room temperature in buffer A (50 mM HEPES pH 8.1, 250 mM KCl) in the presence of 1% Triton X-100 (w/v), $MgCl_2$ (10 mM), PMSF (1 mM), lysozyme (0.5 mg/cell g), DNase I and RNase A (trace). After centrifugation (38,000 x g, 60 min, 4˚C), the *Cr*HydA clarified lysate was purified via FPLC on a 10 mL Strep-tactin®XT high capacity column equilibrated with buffer A. The protein was eluted with 100% buffer B (50 mM HEPES pH 8.1, 250 mM KCl, 50 mM biotin) and reconstituted to increase [4Fe-4S] cluster content. Briefly, purified *Cr*HydA (100 µM) was incubated with DTT (5 mM) for 5 minutes in buffer A, before ferrous ammonium sulfate (0.4 mM final concentration, from a 10 mM stock) and sodium sulfide (0.4 mM final concentration, from a 10 mM stock) were added over the course of an hour. After a 2 h incubation at 21˚C, the solution was centrifuged, desalted (Sephadex G-25 resin column, 75 mL) into buffer A, and concentrated to 188 µM (Amicon 30 kDa MWCO centrifugation filters) to yield a final iron content of 3.9 ± 0.2 irons/protein. EPR spectroscopy after reduction with dithionite confirmed $[4Fe\text{-}4S]^{+}$ cluster presence.

Aminomethyltransferase from *Escherichia coli* preparation

The synthetic *gcvT* gene from *Escherichia coli* was purchased from Genscript™. The DNA sequence was codon-optimized for expression in *Escherichia coli* and was cloned into a pET-23a vector between the *Nde*I and *Xho*I restriction sites, allowing for expression of aminomethyltransferase (T-protein) with a C-terminal His_6 tag in BL21(DE3) *E. coli* cells. The corresponding DNA sequence is as follows:

ATGGCGCAGCAAACCCCGCTGTACGAGCAGCACACCCTGTGCGGTGCGC
GTATGGTGGACTTCCACGGCTGGATGATGCCGCTGCACTATGGTAGCCA
AATCGATGAACACCACGCGGTTCGTACCGACGCGGGCATGTTCGATGTG
AGCCACATGACCATTGTTGACCTGCGTGGTAGCCGTACCCGTGAGTTTC
TGCGTTACCTGCTGGCGAACGATGTGGCGAAGCTGACCAAAAGCGGCA
AGGCGCTGTATAGCGGCATGCTGAACGCGAGCGGTGGCGTGATCGACG
ATCTGATTGTTTACTATTTCACCGAGGACTTCTTTCGTCTGGTGGTTAAC
AGCGCGACCCGTGAAAAGGACCTGAGCTGGATCACCCAGCACGCGGAG
CCGTTTGGCATCGAAATTACCGTGCGTGACGATCTGAGCATGATTGCGG
TTCAGGGTCCGAACGCGCAAGCGAAAGCGGCGACCCTGTTCAACGACG
CGCAGCGTCAAGCGGTGGAAGGCATGAAGCCGTTCTTTGGCGTTCAAGC
GGGTGACCTGTTCATTGCGACCACCGGTTACACCGGTGAAGCGGGTTAT
GAAATTGCGCTGCCGAACGAGAAAGCGGCGGACTTCTGGCGTGCGCTG
GTTGAGGCGGGTGTTAAACCGTGCGGTCTGGGCGCGCGTGACACCCTGC

GTCTGGAGGCGGGCATGAACCTGTACGGTCAGGAGATGGATGAAACCA
TCAGCCCGCTGGCGGCGAACATGGGTTGGACCATTGCGTGGGAACCGGC
GGACCGTGATTTTATTGGTCGTGAGGCGCTGGAAGTGCAACGTGAGCAC
GGCACCGAAAAACTGGTGGGCCTGGTTATGACCGAGAAGGGCGTGCTG
CGTAACGAACTGCCGGTTCGTTTCACCGATGCGCAGGGCAACCAACACG
AGGGTATCATTACCAGCGGCACCTTTAGCCCGACCCTGGGTTATAGCAT
TGCGCTGGCGCGTGTGCCGGAGGGTATTGGCGAAACCGCGATCGTTCAG
ATTCGTAACCGTGAAATGCCGGTGAAAGTTACCAAGCCGGTGTTTGTTC
GTAACGGCAAGGCGGTTGCG

Which leads to the following protein sequence:

MAQQTPLYEQHTLCGARMVDFHGWMMPLHYGSQIDEHHAVRTDAGMFD
VSHMTIVDLRGSRTREFLRYLLANDVAKLTKSGKALYSGMLNASGGVIDDL
IVYYFTEDFFRLVVNSATREKDLSWITQHAEPFGIEITVRDDLSMIAVQGPNA
QAKAATLFNDAQRQAVEGMKPFFGVQAGDLFIATTGYTGEAGYEIALPNEK
AADFWRALVEAGVKPCGLGARDTLRLEAGMNLYGQEMDETISPLAANMG
WTIAWEPADRDFIGREALEVQREHGTEKLVGLVMTEKGVLRNELPVRFTDA
QGNQHEGIITSGTFSPTLGYSIALARVPEGIGETAIVQIRNREMPVKVTKPVFV
RNGKAVAEHHHHHH

After inoculation from overnight starter cultures, cell culture growth in phosphate buffered LB media (9 L total in 6 x 2.8 L Fernbach flasks) supplemented with ampicillin (100 µg/mL) and maintained at 37˚C with shaking (~220 rpm) to an OD_{600} of 0.8. The cultures were chilled to 18˚C, induced with IPTG (1 mM), and incubated overnight at 16 – 20˚C while shaking (~220 rpm). To harvest the cells, the cultures were centrifuged (12,000 x g, 10 min., 4˚C) and the wet cell pellets were immediately frozen in liquid nitrogen and stored at -80˚C. To isolate the T-protein, all lysis and purification steps were performed in an anaerobic Coy vinyl glove box. At a ratio of ~2 mL of buffer per 1 g of cell pellet, lysis was performed at room temperature in buffer A (50 mM HEPES pH 7.5, 250 mM KCl) in the presence of 1% Triton X-100 (w/v), $MgCl_2$ (10 mM), PMSF (1 mM), lysozyme (0.5 mg/cell g), DNase I and RNase A. After centrifugation (38,000 x g, 60 min., 4˚C), the T-protein was purified from the clarified lysate via FPLC on a 5 mL HisTrap column with the following step gradient: 1) 0 % Buffer B (50 mM HEPES pH 7.5, 250 mM KCl, 500 mM imidazole) for 5 CV, 2) 5 % Buffer B for 5 CV, 3) 50 % Buffer B to elute AMT. After brief centrifugation, the enzyme solution was gel filtrated (Sephadex G-25 resin column, 75 mL) with buffer A and concentrated to 600 µM.

Serine hydroxymethyltransferase from *Escherichia coli* preparation

The synthetic *glyA* gene from *Escherichia coli* was purchased from Genscript™. The DNA sequence was codon-optimized for expression in *Escherichia coli* and was cloned into a pET-14b vector between the *Nde*I and *BamH*I restriction sites allowing for expression of SHMT protein with a N-terminal His_6 tag in BL21(DE3) *E. coli* cells. The corresponding DNA sequence is as follows:

ATGCTGAAGCGTGAGATGAACATTGCGGATTATGATGCGGAACTGTGGC
AGGCGATGGAACAAGAAAAAGTGCGTCAAGAGGAACACATCGAGCTGA
TTGCGAGCGAAAACTACACCAGCCCGCGTGTTATGCAAGCGCAGGGTAG
CCAACTGACCAACAAGTACGCGGAGGGTTATCCGGGCAAACGTTACTAT
GGTGGCTGCGAGTATGTGGACATCGTTGAACAGCTGGCGATTGATCGTG
CGAAGGAACTGTTCGGTGCGGATTACGCGAACGTGCAACCGCACAGCGG

CAGCCAGGCGAACTTTGCGGTTTATACCGCGCTGCTGGAGCCGGGTGAC
ACCGTGCTGGGCATGAACCTGGCGCATGGTGGCCACCTGACCCATGGTA
GCCCGGTTAACTTCAGCGGCAAGCTGTACAACATCGTGCCGTATGGTATT
GATGCGACCGGCCACATCGACTACGCGGATCTGGAGAAGCAAGCGAAAG
AACACAAGCCGAAAATGATCATTGGTGGCTTCAGCGCGTATAGCGGTGT
GGTTGACTGGGCGAAAATGCGTGAAATCGCGGATAGCATTGGCGCGTAC
CTGTTTGTTGACATGGCGCATGTTGCGGGTCTGGTTGCGGCGGGCGTGTA
TCCGAACCCGGTGCCGCATGCGCATGTGGTTACCACCACCACCCACAAG
ACCCTGGCGGGTCCGCGTGGTGGCCTGATCCTGGCGAAAGGTGGCAGCG
AGGAACTGTACAAGAAACTGAACAGCGCGGTTTTTCCGGGTGGCCAGGG
TGGCCCGCTGATGCACGTTATTGCGGGCAAGGCGGTGGCGCTGAAAGAA
GCGATGGAGCCGGAATTTAAGACCTACCAGCAACAGGTGGCGAAGAACG
CGAAAGCGATGGTTGAGGTGTTCCTGGAACGTGGCTATAAAGTGGTTAG
CGGTGGCACCGATAACCACCTGTTTCTGGTGGACCTGGTTGATAAGAACC
TGACCGGTAAAGAGGCGGATGCGGCGCTGGGTCGTGCGAACATCACCGT
TAACAAGAACAGCGTGCCGAACGACCCGAAAAGCCCGTTCGTTACCAGC
GGTATCCGTGTGGGTACCCCGGCGATTACCCGTCGTGGTTTTAAAGAGGC
GGAAGCGAAAGAGCTGGCGGGCTGGATGTGCGACGTTCTGGATAGCATT
AACGACGAAGCGGTGATTGAGCGTATTAAGGGCAAGGTTCTGGACATCT
GCGCGCGTTATCCGGTTTATGCGtaa

Which leads to the following protein sequence:

MGSSHHHHHHSSGLVPRGSHMLKREMNIADYDAELWQAMEQEKVRQEEHI
ELIASENYTSPRVMQAQGSQLTNKYAEGYPGKRYYGGCEYVDIVEQLAIDR
AKELFGADYANVQPHSGSQANFAVYTALLEPGDTVLGMNLAHGGHLTHGS
PVNFSGKLYNIVPYGIDATGHIDYADLEKQAKEHKPKMIIGGFSAYSGVVDW
AKMREIADSIGAYLFVDMAHVAGLVAAGVYPNPVPHAHVVTTTTHKTLAG
PRGGLILAKGGSEELYKKLNSAVFPGGQGGPLMHVIAGKAVALKEAMEPEF
KTYQQQVAKNAKAMVEVFLERGYKVVSGGTDNHLFLVDLVDKNLTGKEA
DAALGRANITVNKNSVPNDPKSPFVTSGIRVGTPAITRRGFKEAEAKELAGW
MCDVLDSINDEAVIERIKGKVLDICARYPVYA

After inoculation from overnight starter cultures, cultures were grown in phosphate buffered LB media (9 L total in 6 x 2.8 L Fernbach flasks) supplemented with ampicillin (100 µg/mL) and maintained at 37˚C with shaking (~220 rpm) to an OD_{600} of 0.8. The cultures were chilled to 16 – 20˚C, induced with IPTG (1 mM), and incubated overnight at 16 – 20˚C while shaking (~220 rpm). To harvest the cells, the cultures were centrifuged (12,000 x g, 10 min, 4˚C) and the wet cell pellets were immediately frozen in liquid nitrogen and stored at -80˚C. To isolate SHMT, all lysis and purification steps were performed in an anaerobic Coy vinyl glove box. At a ratio of ~2 mL of buffer per 1 g of cell pellet, lysis was performed at room temperature in buffer A (50 mM HEPES pH 7.5, 250 mM KCl) in the presence of 1% Triton X-100 (w/v), $MgCl_2$ (10 mM), PMSF (1 mM), lysozyme (0.5 mg/cell g), DNase I and RNase A. After centrifugation (38,000 x g, 60 min., 4˚C), the SHMT was purified from the clarified lysate via FPLC on a 5 mL HisTrap column with the following step gradient: 1) 0 % Buffer B (50 mM HEPES pH 7.5, 250 mM KCl, 500 mM imidazole) for 5 CV, 2) 5 % Buffer B for 5 CV, 3) 50 % Buffer B to elute SHMT. After brief centrifugation, the enzyme solution was gel filtrated (Sephadex G-25 resin column, 75 mL) with buffer A and concentrated to ~1 mM.

P-protein from *Synechocystis sp.* PCC 6803 preparation

The *Synechocystis* P-protein preparation was performed as described previously[5] with minor modifications. The protein sequence is as follows:

MGGSHHHHHHGMASMTGGQQMGRDLTDDDDKDRWGSEMPNLEPAVVVP
TSEAIAVDLTKLEEKLAPADSFLDRHLGPGETEQRQMLQTLGFDTLGDLIDQ
AVPPAIRFPRSLQLPASQSEYGAIAQLKSIASKNQVFRSYIGMGYYDTITPPVI
QRNILENPGWYTAYTPYQAEIAQGRLEALLNFQTMVMDLTGLEIANASLLD
EGTAAAEAMALSYGVSKSKANAFFVAQDCHPQTIEVIKTRANPLGIEVIVGD
HHTFSFSTSIFGALLQYPATDGAVYDYRSFIDKAHQHQALVTLAADPLSLTL
LTPPGELGADIAVGSTQRFGIPLGYGGPHAAYFATKAEYQRKMPGRIVGVSK
DAHGNPALRLALQTREQHIRRDKATSNICTAQVLLAVMASMYGVYHGSTG
LKNIALRIHQLTVLLAIGLKRLNYSLNNDYFFDTLRVGVGEQSAPAILKAAE
GRGINLRPLVPGEVGISLDETVTVQDLLDLWQVFAGKDNLPFTPEELWSEVK
TSFPADLTRQSLYLQDAVFNQYHSETELLRYLHQLESKDLALNTSMIPLGSC
TMKLNATAEMMPVTWPEFGKIHPFAPAGQTEGYQILFAQLEAWLGEITGFD
AISLQPNAGSQGEYAGLQVIRQYHLSRGEEQRNICLIPESAHGTNPASAVMC
GMQVVPVKCDGEGNIDVEDLTSKAEKYGDRLAALMVTYPSTHGVFEATIGT
ICDIVHRFGGEVYMDGANMNAQVGLCRPADFGADVCHLNLHKTFCIPHGG
GGPGMGPIGVKSHLQAFLPRTSLNSTAELQAEDQSIGMISAAPYGSASILVIS
WMYIAMMGPQGLTKATEVAILSANYMAKRLENYYPILFRGNNELVAHECIL
DLRPLKKQAAIEVEDVAKRLMDFGFHAPTVSWPVLGTMMVEPTESESLGEL
DRFCDAMIAIYQEAQAITHGEIDPADNPLKNAPHTAQSLICGEWNHPYSQEE
AAYPAPWTKQFKFWPAVGRINNTYGDRHLVCSCEGMEAYKEG

The recombinant *slr0293* gene from *Synechocystis* sp. PCC 6803 was expressed using an *E. coli* BL21 strain containing the pBAD-HisA-slr0293 plasmid. After inoculation from overnight starter cultures, cultures were grown in phosphate buffered LB media (9 L total in 6 x 2.8 L Fernbach flasks) supplemented with ampicillin (100 µg/mL) and maintained at 37˚C with shaking (~220 rpm) to an OD_{600} of 0.8. The cultures were chilled to 18˚C, induced with L-arabinose (0.2 %), and incubated overnight at 18˚C while shaking (~220 rpm). To harvest the cells, the cultures were centrifuged (12,000 x g, 10 min, 4˚C) and the wet cell pellets were immediately frozen in liquid nitrogen and stored at -80˚C. To isolate the P-protein, all lysis and purification steps were performed aerobically. At a ratio of ~2 mL of buffer per 1 g of cell pellet, lysis was performed at room temperature in buffer A (50 mM HEPES pH 7.5, 250 mM KCl) in the presence of 1% Triton X-100 (w/v), $MgCl_2$ (10 mM), PMSF (1 mM), DNase I and RNase A with a microfluidizer at 15,000 PSI. After centrifugation (38,000 x g, 60 min, 4˚C), the P-protein was purified from the clarified lysate via FPLC on a 5 mL HisTrap column with the following step gradient: 1) 0 % Buffer B (50 mM HEPES pH 7.5, 250 mM KCl, 500 mM imidazole) for 5 CV, 2) 5 % Buffer B for 5 CV, 3) 60 % Buffer B to elute P-protein. After brief centrifugation, the enzyme solution was gel filtrated (Sephadex G-25 resin column, 75 mL) with buffer A and concentrated to 33 µM.

H-protein from *Escherichia coli* preparation

The expression and purification of his-tagged H protein in its apo form followed previously published procedures.[6]

Synthesis of Lipoyl-H-Protein
The reaction mixture contained, in a final volume of 50 mL, 50 mM potassium phosphate buffer (KPB), pH 7.2, 15 mM $MgCl_2$, 5 mM ATP, 10 mM TCEP-HCl, 20 mM DTT, 5 mM lipoic acid, 200 µM apo H protein and 100 nM lipoate protein ligase A (LplA). The reaction was allowed to incubate at 28 °C for 5 h, upon which it was diluted 10x with 20 mM KPB, pH 7.2. The diluted protein was loaded onto a DE-52 column that was pre-equilibrated in the same buffer. The column was washed with 300 mL 10 mM KPB, pH 7.2 containing 180 mM NaCl and then eluted with a 600 mL (total) linear gradient of 180 - 450 mM NaCl in 20 mM KPB, pH 7.2. The lipoylated H protein was concentrated to a minimal volume and buffer-exchanged into storage buffer (50 mM HEPES pH 7.5, 250 mM KCl and 30% glycerol) using a PD-10 gel-filtration column. The protein was aliquoted, snap-frozen in liquid N_2 and stored at -80 ºC until needed.

Loading lipoyl-H-protein with methylamine via P-protein
The loading of lipoyl-H-protein with methylamine via the P-protein was performed aerobically as described previously[7] with minor modifications. The 5 mL reaction in 20 mM potassium phosphate pH 7.4, contained 64 µM lipoyl-H-protein, 4.5 µM dimeric P-protein, 64 mM glycine and 64 µM PLP. After a 2 h incubation exposed to air at room temperature, the reaction mixture was loaded inside a Coy chamber onto a HiPrep 16/60 Sephacryl S-200 High Resolution column equilibrated with anaerobic buffer (50 mM HEPES pH 6.5, 250 mM KCl). Fractions containing aminomethyl-lipoyl-H-protein were combined and concentrated to 1.2 mM. Generation of the aminomethyl-lipoyl-H-protein was verified by MS (**Figure S3**).

SAM preparation
SAM preparation was performed as described previously.[8]

Purification of holo-*Cr*HydA
Strep-Tactin affinity purification was carried out after maturation in an anaerobic MBraun glovebox with 100% N_2 environment. Strep-tagged mature *C.r.* HydA (holo-HydA) was re-purified from the maturation reaction mixture through a 2.5 mL Strep-Tactin XT column. 50 mM HEPES pH 8.0, 150 mM KCl (wash buffer) and 50 mM HEPES pH 8.0, 150 mM KCl with 50 mM D-biotin (elution buffer) were prepared and degassed using a Schlenk-line. Strep-Tactin XT column (2.5 mL resin bed) was equilibrated with 10 column volumes (CV) of 50 mM HEPES pH 8.0, 150 mM KCl with freshly added 1 mM sodium dithionite (NaDT). 20 mL maturation mixture was loaded to the equilibrated column, followed by a wash step with 50 mM HEPES pH 8.0, 150 mM KCl (1 mM fresh NaDT added) for 5 CV. Holo-HydA was eluted in 50 mM HEPES pH 8.0, 150 mM KCl, 50 mM D-biotin with 1 mM NaDT. The dark colored elution fraction was then concentrated using Amicon Ultra centrifugal filters (MWCO 30 kDa).

Verification of Protein Purity
All proteins used in the in vitro maturation reactions, including the HydA, the maturases HydE, HydF, and HydG, and the components of the GCS (H-protein, AMT, and SHMT) were verified to be pure by using SDS-PAGE (**Figure S4**).

EPR and ENDOR Sample Preparation
X-band EPR samples were prepared with freshly purified holo-HydA in an MBraun glovebox with 100% N_2 atmosphere. Matured holo-HydA was mixed with 2 mM thionin acetate from a

freshly prepared stock solution and incubated for 2.5 min, then transferred into an EPR tube, capped with a rubber septum, and then immediately transferred from the chamber and flash frozen in liquid N_2. To make H_{ox} ENDOR samples, holo-HydA was loaded into Q-band tubes, oxidized with 2 mM thionin, capped with rubber septa, and then immediately transferred from the MBraun chamber and flash frozen in liquid N_2. Samples were stored in liquid N_2 until spectral acquisition occurred.

Mass spectrometry

H_{met} intact protein samples (10 μM) were analyzed using a 1290 ultrahigh pressure (UPLC) series chromatography stack (Agilent Technologies) coupled directly to an electrospray-time of flight (ESI-TOF) mass spectrometer (Bruker Micro-TOF). Before infusion to the ESI source, sample was injected on a size exclusion (SEC) Polyhydroxyethyl A column (100 × 4.6 mm, 5 μm, 500 Å, PolyLC Inc.) with flow rates of 300 μL/min (SEC column). Isocratic conditions (25% B) were used to elute protein from the SEC column (temperature of 25 °C, 4 min). Solvent A comprised 0.1% formic acid (FA, Sigma) in water (ThermoFisher) and solvent B comprised 0.1% FA in acetonitrile (ThermoFisher). Electrospray conditions were as follows: nebulizer 3.5 bar, drying gas at a flow rate of 6.0 L/min, drying temperature at 200 °C, capillary voltage at 4.5 kV, and capillary exit voltage at 150 V. Data was collected in positive mode only at 2 Hz rate over the 200–3000 *m*/*z* scan range. Data processing and analysis were performed using the Bruker Data Analysis package 4.0. Charge deconvolution was performed using a maximum entropy algorithm for H+ adducts only and 0.1 *m*/*z* data point spacing. Measured *m*/*z* errors for the protein were less than 0.8 *m*/*z* from calculated values.

***In vitro* activation assays**

The *in vitro* activation assays were performed as described previously with modifications.[3] *In vitro* maturations of *Cr*HydA were carried out at ambient temperature in an anaerobic MBraun chamber (O_2 ≤1 ppm) with or without the GCS proteins. A standard reaction without the GCS proteins consisted of 25 μM HydG (reconstituted, containing 8–9 Fe per protein), 5 μM HydF (4 Fe per protein), 5 μM HydE (8 Fe per protein), 4 μM HydA (4 Fe per protein), 2 mM L-tyrosine, 2 mM L-cysteine, 2.5 mM SAM, 1 mM DTT, 2 mM DT, 20 mM GTP, and 1.6 mM Fe^{2+} as ferrous ammonium sulfate (FAS). Assay components were incubated together (200 μL final volume) for 12 hours in a 100 mM HEPES, pH 8.2, 50 mM KCl buffer, prior to removing an aliquot to assay for active hydrogenase. For the assays in Figure 2, either no H-protein, or purified lipoyl-H-protein (20 μM) or purified aminomethyl-lipoyl-H-protein (20 μM or 600 μM) was added; no *E. coli* lysate was added. The assays in Figure 3 contain HydA (4 μM), HydE (5 μM), HydF (5 μM), HydG (25 μM), Fe(II) (6.4 mM), L-cysteine (2 mM), L-tyrosine (2 mM), SAM (2.5 mM), GTP (20 mM), dithionite (2 mM), PLP (1 mM), DTT (1 mM), T-protein (10 μM), and SHMT (5 μM). Serine and NH_4Cl were added either to 50 mM or at the concentrations indicated. Glycine and THF were added at the concentrations indicated. Reactions contained no lysate. We found that in maturation reactions containing the T-protein, addition of the H-protein was no longer necessary due to co-purification of the H-protein with the T-protein;[9] adding additional H-protein to these assays in Figure 3 does not change maturation, indicating that sufficient H-protein copurifies to support maturation.

To prepare the hydrogenase activity assay, 2 μL of the reaction mixture was diluted to 2 mL (final volume) using 50 mM Tris, pH 6.9, 10 mM KCl buffer; DT and methyl viologen were then

added to the mixture to final concentrations of 20 mM and 10 mM, respectively. After 3 minutes, headspace gas (100 μL) was removed from the vial with a Hamilton gas tight syringe; H_2 production was quantified using a SHIMADZU GC-2014 with a TCD detector, with N_2 as a carrier gas.

EPR and ENDOR Spectroscopy

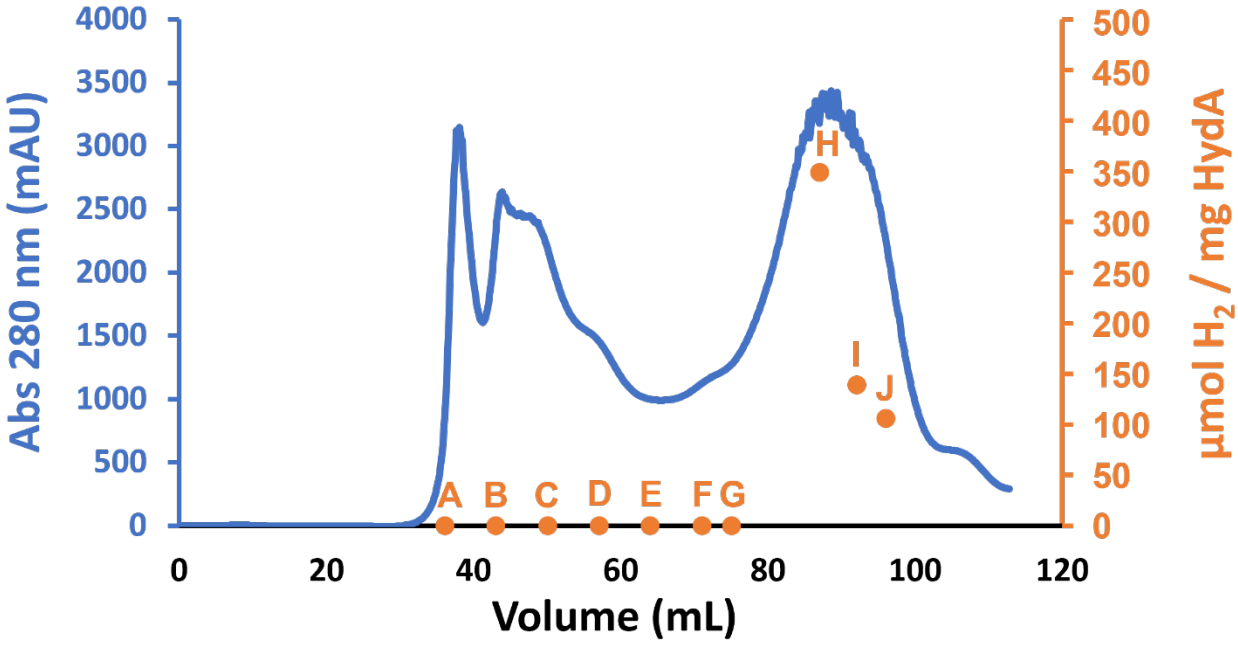
Low temperature continuous wave (CW), X-band (9.38 GHz) EPR spectra were collected using a Bruker EMX spectrometer fitted with a ColdEdge (Sumitomo Cryogenics) 10 K waveguide in-cavity cryogen free system, with Oxford Mercury iTC controller unit and helium Stinger recirculating unit (Sumitomo Cryogenics, ColdEdge Technologies, Allentown, PA). Helium gas flow was maintained at 100 psi. Spectral parameters for H_{ox} data collection were: 1.0 mW microwave power, 100 kHz modulation frequency, 10 G modulation amplitude, and spectra were averaged over 4 scans. Spectral data were plotted using the software program OriginPro (2018b, OriginLab Corp. Northampton, MA, USA). 35 GHz pulse ^{13}C and ^{15}N ENDOR spectra were collected on a spectrometer described previously,[10] that is equipped with a helium immersion dewar for measurement at 2K. For a single molecular orientation and for nuclei with nuclear spin of $I = {}^1/_2$ (^{15}N, ^{13}C), the ENDOR transitions for the $m_s = \pm{}^1/_2$ electron manifolds are observed, to first order, at frequencies, where ν_n is the nuclear Larmor frequency, and A is the orientation-dependent hyperfine coupling:

$$I = ½: \upsilon_{\pm} = \left| \upsilon_{\mathrm{n}} \pm \frac{A}{2} \right|$$

In the Mims experiment, the ENDOR intensities are modulated by an inherent response factor (R),

$$R \sim [1 - \cos(2\pi A\tau)] \quad (2)$$

where τ is the interval between the first and second pulses in the three-pulse Mims spin-echo sequence. When $A\tau = n$ ($n = 0, 1, 2\ldots$), the ENDOR response is at a minimum, resulting in hyperfine 'suppression holes' in the Mims spectra. In **Fig 4** these holes are indicated by (↓).



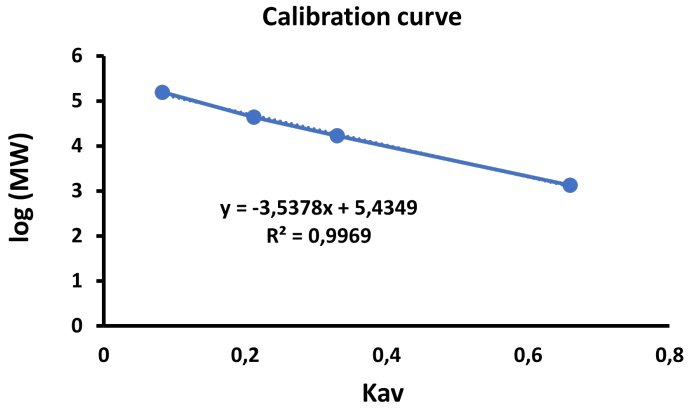


Figure S1. Gel filtration of *E. coli* cell lysate as monitored by absorbance at 280 nm (blue). Individual fractions were used in a hydrogenase maturation assay to assess their ability to support maturation (orange data points and right-hand axis). Fractions H, I, and J, which eluted in the 10 – 15 kDa MW region of the profile, were able to support maturation of $HydA^{\Delta EFG}$. Assays for H_2 were run for 24 h to allow H_2 to accumulate. Lower panel shows the calibration curve for the column.

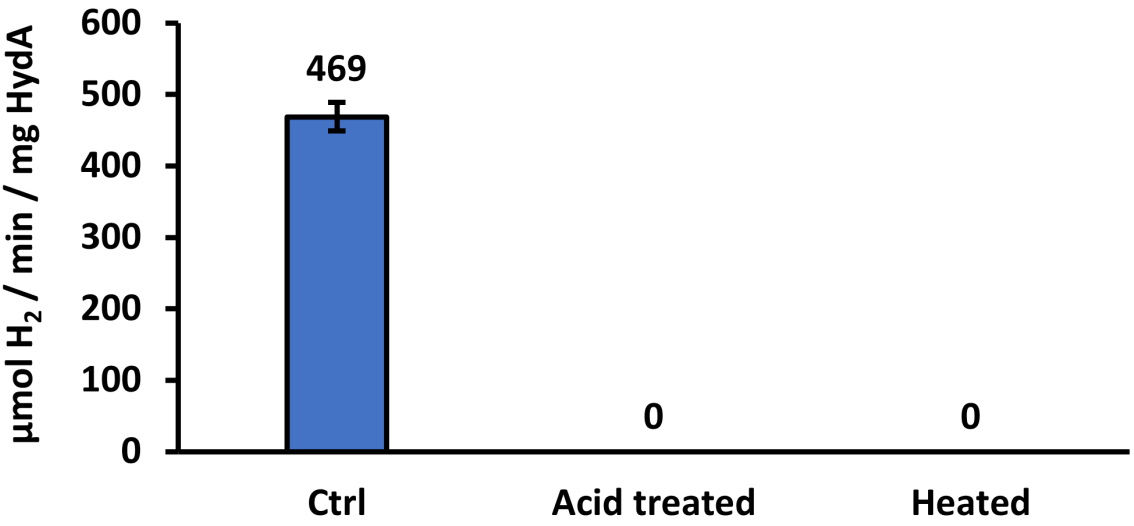


Figure S2. *E. coli* cell lysate supports *in vitro* maturation of the [FeFe]-hydrogenase in the presence of the maturases HydE, HydF, and HydG, as evidenced by the activity of the resulting active [FeFe]-hydrogenase (Ctrl). If the *E. coli* lysate is first treated with acid (middle) or heat (right) before being added to the *in vitro* maturation reaction, no active HydA is produced.

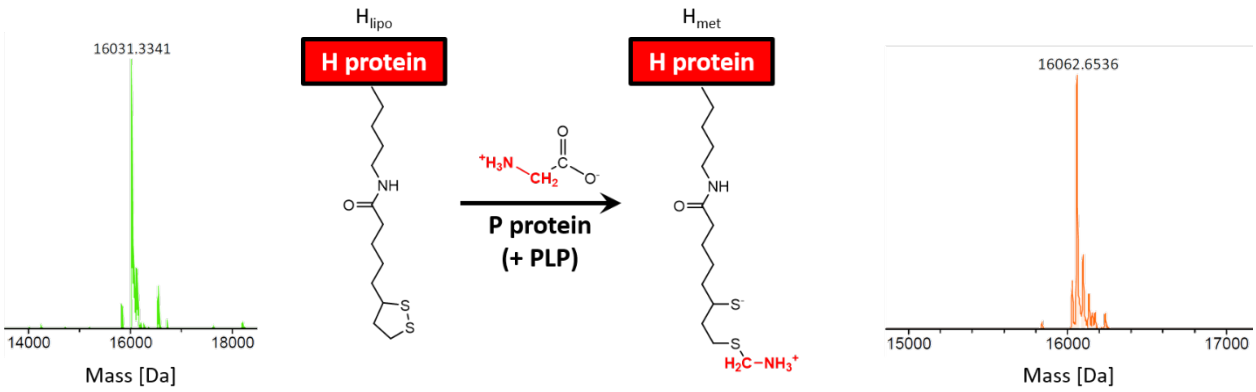


Figure S3. Loading of the lipoyl-H protein with the aminomethyl group by using the P-protein and glycine, as monitored by mass spectrometry.

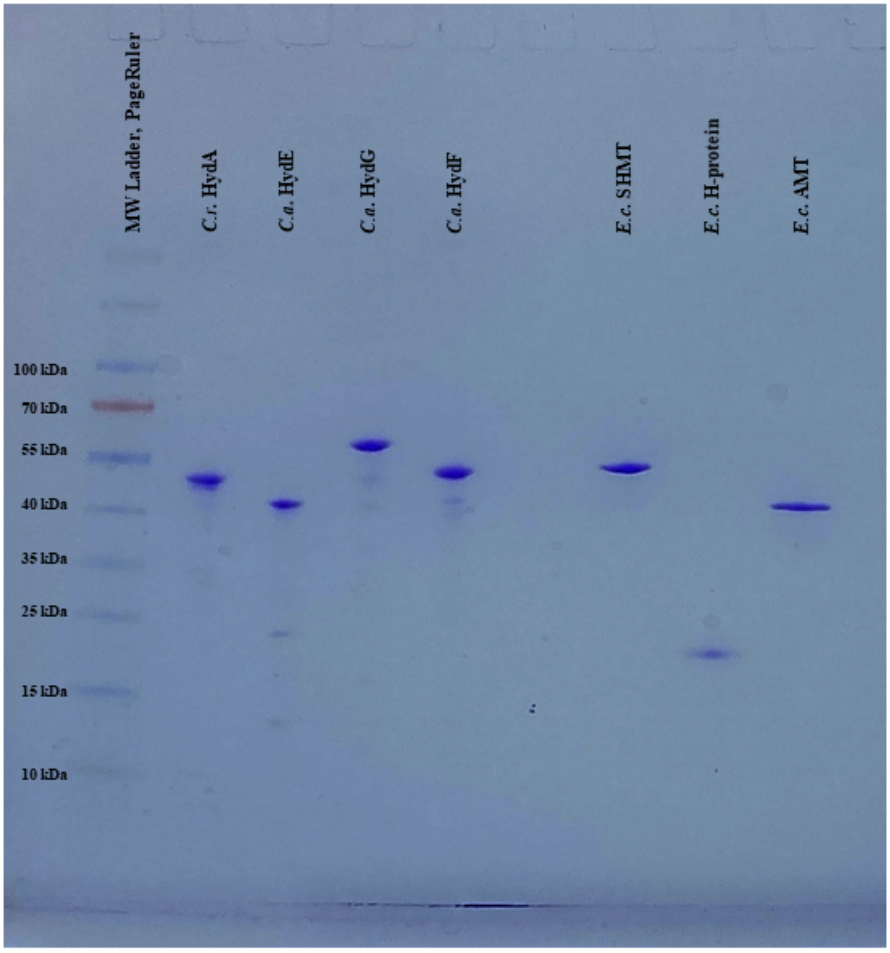


Figure S4. SDS-PAGE gel of purified proteins used in this study: *Chlamydomonas reinhardtii* (*C.r.*) HydA, *Clostridium acetobutylicum* (*C.a.*) HydE, HydF, and HydG, and *Escherichia coli* (*E.c.*) serine hydroxymethyltransferase (SHMT), H-protein, and aminomethyltransferase (AMT, T-protein).

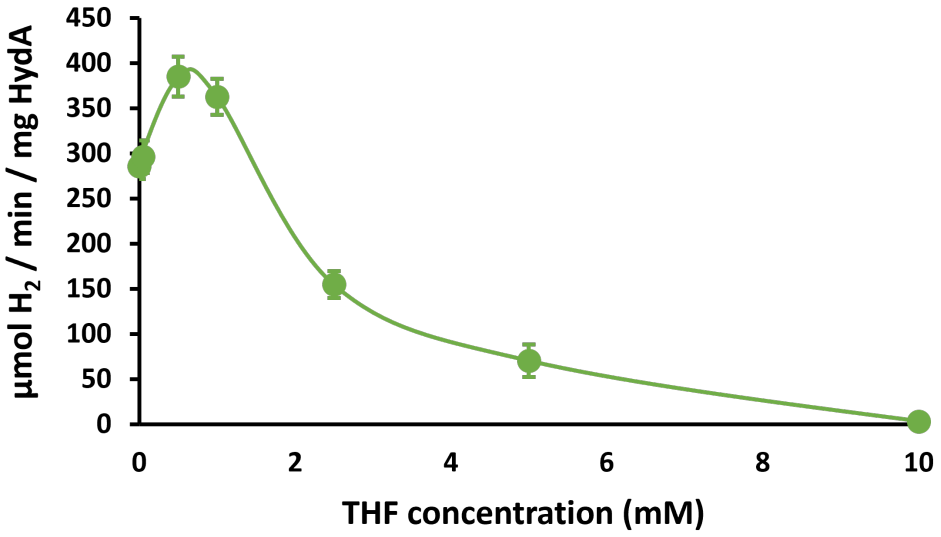


Figure S5. Dependence of HydA maturation on THF concentration, zooming in on the lower concentration region plotted in **Figure 3**.

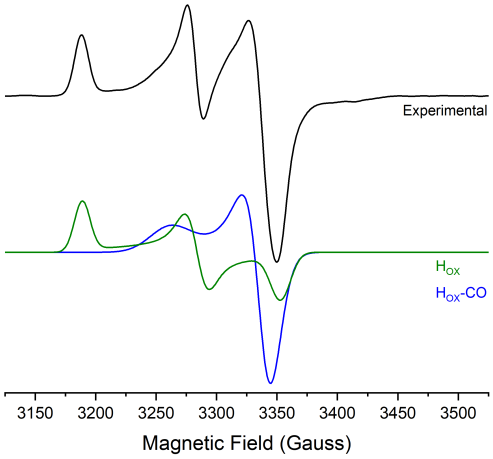
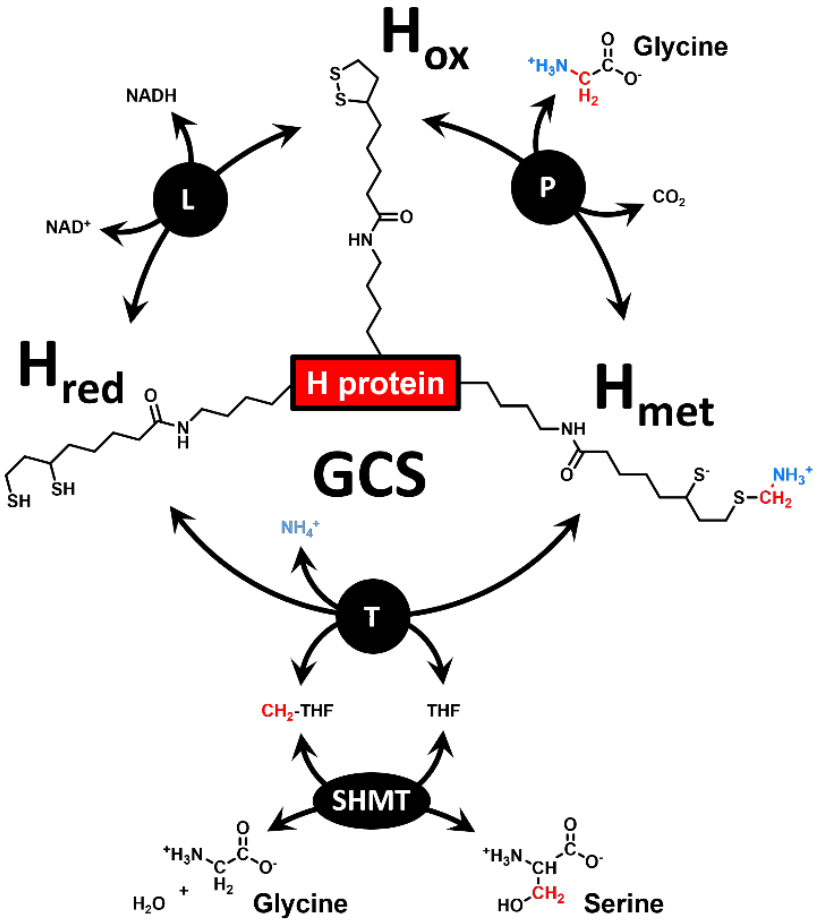


Figure S6. X-band EPR spectroscopy of HydA (150 μM, black) matured in the absence of lysate, but in the presence of the T-protein, SHMT, ammonium, and serine, purified from the maturation reaction, and then oxidized with 2 mM thionin. EPR parameters: 12 K, 1 mW microwave power, 10 G modulation amplitude. The spectrum was simulated as a mixture of H_{ox} (green, g-values of 2.100, 2.039, and 1.998) and H_{ox}-CO (blue, g-values of 2.055, 2.007, 2.007). for the experimental signal. Simulations were performed using the EasySpin software program.[11]



Scheme S1. The glycine cleavage system (GCS) and serine hydroxymethyltransferase (SHMT) as discussed in the main text. The lipoyl-H-protein (center) acts as a carrier between the three GCS enzymes: aminomethyltransferase (T-protein, bottom), dihydrolipoyl dehydrogenase (L-protein, upper left), and glycine dehydrogenase (P-protein, upper right). SHMT is coupled to the T-protein through the common substrates/products tetrahydrofolate (THF) and methylene-tetrahydrofolate (CH_2-THF). The double-headed arrows are used to show that all reactions are reversible. H_{met} can be formed either from H_{ox} and glycine via the P-protein, in which case the aminomethyl moiety is derived from glycine. Alternatively under reducing conditions where H_{red} is the predominant form of the lipoyl-H-protein, H_{met} can be formed from H_{red} via the T-protein, wherein the aminomethyl moiety is derived from serine and ammonium.

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