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To cite this version:

Giorgio Caserta, Cécilia Papini, Agnieszka Adamska-Venkatesh, Ludovic Pecqueur, Constanze Sommer, et al.. Engineering an [FeFe]-hydrogenase: do accessory clusters influence O$_2$ resistance and catalytic bias?. Journal of the American Chemical Society, American Chemical Society, 2018, 140, pp.5516-5526. 10.1021/jacs.8b01689. hal-01759288

HAL Id: hal-01759288
https://hal-amu.archives-ouvertes.fr/hal-01759288
Submitted on 25 May 2018

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Engineering an [FeFe]-hydrogenase: do accessory clusters influence O₂ resistance and catalytic bias?

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Keywords: hydrogenase, Megasphaera elsdenii, H cluster, O₂ sensitivity, catalytic bias

Abstract: [FeFe]-hydrogenases, HydAs, are unique biocatalysts for proton reduction to H₂. However, they suffer from a number of drawbacks for biotechnological applications: size, number and diversity of metal cofactors, oxygen sensitivity. Here we show that HydA from Megasphaera elsdenii (MeHydA) displays significant resistance to O₂. Furthermore, we produced a shorter version of this enzyme (MeH-HydA), lacking the N-terminal domain harbouring the accessory FeS clusters. As shown by detailed spectroscopic and biochemical characterization, MeH-HydA displays the following interesting properties. First, a functional active site can be assembled in MeH-HydA in vitro, providing the enzyme with excellent hydrogenase activity. Second, the resistance of MeHydA to O₂ is conserved in MeH-HydA. Third, MeH-HydA is more biased towards proton reduction than MeHydA, as the result of the truncation changing the rate limiting steps in catalysis. This work shows that it is possible to engineer HydA to generate an active hydrogenase that combines the resistance of the most resistant HydAs and the simplicity of algal HydAs, containing only the H-cluster.

Introduction

Water splitting to hydrogen and oxygen is one of the few promising strategies for storing renewable, intermittent and diluted energies at large scale. In particular, the development of industrially relevant energy conversion (photo)-electrochemical systems, such as electrolyzers, photoelectrochemical cells and fuel cells, will depend on our ability to use earth-abundant elements exclusively and to optimize cheap, stable and efficient catalysts for the redox reactions at the electrodes. Current research in this direction targets both synthetic solid and molecular catalysts. However, enzymes are also extensively studied (e.g. for use in bioelectrodes) since they display remarkable performances in particular with respect to turnover frequencies (TOFs), far exceeding those displayed by synthetic catalysts. This is especially true for hydrogenases which catalyze the interconversion of molecular hydrogen into protons and electrons with TOF values up to 10⁴ s⁻¹.¹ These enzymes operate under mild conditions and close to the thermodynamic reaction equilibrium, i.e. with very little overpotential. There
are two classes of hydrogenases, the [NiFe]-hydrogenases and the [FeFe]-hydrogenases, based on the metal content of their respective active site. The work presented here deals exclusively with the second class.

Only two fully active forms of [FeFe]-hydrogenases, named HydAs in the following, have been structurally characterized so far: HydA from *Clostridium pasteurianum* (CpHydA) and HydAB from *Desulfovibrio desulfuricans* (DdHydAB). These studies have established that the enzyme active site (referred to as the H-cluster) consists of a diiron center (referred to as the 2Fe-subcluster) sharing a cysteine ligand with a [4Fe-4S] cluster (Scheme 1). Each Fe atom of the 2Fe-subcluster has one CO and one CN– ligand and is connected to the other through a bridging CO ligand. The coordination sphere is completed by a unique azapropanedithiolate (adt²–) ligand bridging the two Fe atoms. Most HydAs contain accessory [4Fe-4S] and [2Fe-2S] ferredoxin-like clusters that electronically connect the active site to the protein surface. For example, clostridial HydAs contain four accessory [Fe-S] centers while DdHydAB and *Megasphaera elsdenii* HydA (MeHydA) contain two ferredoxin-like [4Fe-4S] clusters. In contrast, [FeFe]-hydrogenases from green algae, such as *Chlamydomonas reinhardtii* (CrHydA), only contain the catalytic H-cluster and no accessory clusters, thus representing the simplest form of [FeFe]-hydrogenases yet identified. So far no three-dimensional structures of fully mature members of that specific class have been obtained.

Scheme 1: Schematic representation of the active site of [FeFe]-hydrogenases. The dashed circle indicates the vacant coordination position on the distal iron of the 2Fe-subcluster.

Despite their high catalytic efficiency, [FeFe]-hydrogenases are nevertheless fraught with a number of drawbacks which still need to be addressed before they can be implemented into biotechnological devices. The first one resides in the complexity of their metal centers and the need for complex protein machineries for their expression and maturation. Recently, we have discovered an efficient chemical maturation technology which provides an exquisite solution to this issue. The second one resides in the limited tolerance of HydAs to molecular oxygen, which varies significantly from one enzyme to another. Current research aims at understanding the molecular basis of the reaction of HydA with O₂ and at finding strategies for improving stability. The third one concerns the size of HydA proteins, which severely limits enzyme density at the surface of the electrodes. In this context, CrHydA, the smallest enzyme, is an interesting hydrogenase. Unfortunately, it belongs to the most O₂-sensitive ones.

So far, all these issues have been addressed using a very limited set of HydAs, almost exclusively CrHydA, CpHydA and, to a lesser extent, DdHydAB. Having recently invested significant efforts in the heterologous expression and characterization of MeHydA, we have studied its reactivity towards O₂ using a previously developed electrochemical method and found it to be, when adsorbed to an electrode, the most O₂-resistant HydA reported so far, through direct experimental comparison with CrHydA and the enzyme from *Clostridium acetobutylicum* CaHydA. Furthermore, considering the great O₂-sensitivity of CrHydA, it has been sometimes suggested that the accessory clusters might contribute to protect the active site from O₂ attack via delivering electrons to the active site. Thus, we also report the results obtained with the first fully characterized and active truncated form of HydA from *Megasphaera elsdenii* (named MeH-HydA), in which the ferredoxin domain has been deleted and thus containing the H-cluster exclusively. While retaining a well-assembled and active H-cluster, MeH-HydA displays specific electrochemical properties. Interestingly, the O₂-resistance of MeH-HydA is retained in MeH-HydA, so that MeH-HydA displays greatly reduced reactivity with respect to O₂ as compared to CrHydA. Furthermore, the truncation results in an enzyme much more biased towards H₂ production, raising new hypothesis regarding the role of the accessory clusters in tuning the bias of HydAs.

Methods

MeHydA was purified, reconstituted and activated as previously described. [Fe₂(adt)(CO)₆] (adt²– = 2-azapropane-1,3-dithiolate) and [Fe₂(ptd)(CO)₆] (ptd²– = propane-1,3-dithiolate) were synthesized according to literature procedures. Preparation of CrHydA was performed as previously described. Protein concentrations were determined with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

Preparation of CaHydA

*E. coli* MG1655ΔiscR::kan strain was obtained by generalized P1 phage-transduction from the JW2515-3 strain from Keio Collection (ECK2528). Removal of kanamycin resistance cassette was by Flp recombinase expressed from pCP20 plasmid leaving an FRT scar and yielding the *E. coli* MG1655ΔiscR::FRT strain.

pthlA-CaHydA-LL-C-Tag plasmid was constructed by replacing the PH (C. acetobutylicum) promoter by the thlA (C. acetobutylicum) thiolase promoter from pTHydA-Cg-C-tag in the pPH_CaHydA-LL-C-Tag plasmid.
C. acetobutylicum maturation proteins genes (hydE, hydF and hydG genes) were PCR amplified from C. acetobutylicum ATCC 824 genomic DNA and further cloned as a single operon in the pACYC184 plasmid (Gene bank accession X06403) under the P₄ promoter dependence, yielding to the custom pACYC_Mat plasmid.

Constructed plasmids and newly generated operons were validated by restriction profile and sequencing, respectively, before to be introduced in the E. coli MG1655ΔiscR::FRT strain.

CaHydA expression was performed with E. coli MG1655ΔiscR::FRT strain containing both pthIA-CaHydA-LL-C-Tag and pACYC_Mat plasmids. This strain was used since overexpression of the isc operon (by deleting the iscR negative regulator) stimulated the Fe-S cluster assembly process leading to higher specific and total recombinant hydrogenase activity. The recombinant strain was grown in custom “MAC” medium (Tryptone 10 g/L, Yeast extract 5 g/L, HEPES 23 g/L, nitrolitriatic acid 0.2 g/L, NaCl 2 g/L, K₂HPO₄ 0.5 g/L, FeSO₄ 0.05 g/L, pH 7.3), packed in 500 mL bottles and sparged with pure nitrogen for 40 minutes before sterilization. Medium was supplemented with glucose (20 g/L final), ammonium ferric citrate (2 mM final), sodium fumarate (25 mM final), sodium nitrate (10 mM final), cysteine (0.32 g/L final) and carbencillinicilin (100 µg/mL final) and chloramphenicol (30 µg/mL final) prior to inoculation. Cultures were inoculated at 0.05 OD₆₀₀nm and incubated at 30°C. Cultures reaching OD₆₀₀nm > 3 (after around 16 hours) were transferred to an anaerobic Jacomex glove box filled with 100% N₂ atmosphere. Cells were collected by centrifugation at 8000 g for 10 min at 4°C, resuspended in buffer (Tris HCl 100 mM, NaCl 150 mM, Glycerol 10%, pH 7.6, DTT 2 mM) and concentrated 30 times. Cells were either stored at -20°C or directly used for enzyme purification.

Protein extraction, hydrogenase purification and purified enzyme purity control were finally performed as previously described.

Preparation of MeH-HydA

Molecular biology. The pT7-7-derived plasmid containing the whole hydrogenase gene (pT7-7-Me-HydA) was used to amplify by PCR and clone into pT7-7 the gene fragment encoding the 405 amino acid residues. This gene fragment lacks the N-terminal part which harbors the ferredoxin domain (methionine 1 to valine 80). The PCR fragment was purified with the High Pure PCR kit (Roche Applied Science), double-digested with NdeI and PstI (Thermo Fisher Scientific) and gel-purified before direct cloning into the pT7-7 expression vector, leading to the pT7-7-MeH-HydA plasmid. Chemically competent DH5α cells were transformed with the plasmid and gene integrity was verified by DNA sequencing.

Expression and purification of MeH-HydA. TunerDE3 cells transformed with the pT7-7-MeH-HydA plasmid were grown in Terrific Broth medium supplemented with ampicillin at 37 °C, until the optical density at 600 nm reached 0.5. Protein synthesis was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were grown for an additional 5-6 h at 20 °C to avoid the formation of inclusion bodies. Cells were harvested by centrifugation and stored at -80°C until use. Cells were re-suspended in Tris–HCl Buffer 50 mM pH 8.0 containing 300 mM NaCl, 0.5 % v/v Triton and discontinuously sonicated for 10-12 min in a water/ice bath. Cellular extracts were centrifuged for 1h at 193000 g and the soluble fraction was loaded on a HisTrap crude FF column (GE-healthcare) equilibrated with 50 mM Tris–HCl pH 8.0, 300 mM NaCl (Buffer A). After extensive wash with 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, the protein was eluted with a linear gradient of Buffer A supplemented with 500 mM imidazole. Fractions containing apo-MeH-HydA were pooled after the addition of 5 mM DTT, concentrated by ultrafiltration and loaded on a Superdex S200 16-600 equilibrated in 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 % w/v glycerol, 5 mM DTT. Only fractions corresponding to the monomer were combined and concentrated giving a pure and homogeneous protein. Finally, the apo-protein was concentrated to 5-10 mg/ml, flash-frozen in liquid nitrogen and stored at -80 °C until use.

Reconstitution of the [4Fe-4S] cluster. The iron-sulfur cluster reconstitution was conducted under strictly anaerobic conditions in a glove box (M Braun) with less than 0.5 ppm O₂. After incubation of apo-protein (100 µM) with 10 mM DTT for 15-20 min at 20°C, a 5-5.5 molar excess of [(NH₄)₂Fe(SO₄)₂]₆H₂O and L-cysteine was added, followed by the addition of a catalytic amount of the E. coli cysteine desulfurase CsdA (1–2 % molar equivalent). Cysteine desulfurase was prepared as reported. The reaction was performed overnight and the cluster assembly reaction was monitored by recording UV–visible absorption spectra every 20 min. The reconstituted protein, called FeS-MeH-HydA, was then centrifuged 20 min at 12000 rpm and purified on Superdex S200 10/300 GL equilibrated with the reconstitution buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 % w/v glycerol) supplemented with 5 mM DTT. A pure and homogeneous fraction was collected, concentrated with Amicon Ultra 30-kDa centrifugation filters (Millipore) and stored at -80 °C in sealed vials under nitrogen atmosphere until use. For Mössbauer characterization the same procedure was followed but using ⁵⁷Fe(SO₄) as a source of Fe, a 0.62 M solution of ⁵⁷Fe(SO₄) in sulfuric acid diluted to 0.023 M in 1.0 M Tris-HCl buffer pH 8.0. For each sample, the Fe and S content was determined 3 times for 2 different protein concentrations according to the methods of Fish²³ and Beinert²⁹ respectively.

Activation of MeH-HydA. In a standard experiment, protein solutions were extensively washed with 100 mM potassium phosphate pH 6.8 to remove DTT traces and avoid DTT-dependent decomposition of the organometallic chemicals. Initially, maturation of FeS-MeH-HydA was conducted as reported for the full length protein: 50 µM protein solution in 100 mM potassium phosphate
pH 6.8 was incubated for 1h with 3-10 equivalents of [Fe2(adt)(CO)4(CN)2]2–. Excess of the chemical was removed using a desalting column (NAP-10, GE healthcare). Maturation was verified by assaying the hydrogenase activity of the protein. A specific activity of 23 ± 3.5 µmol H2.mg-1.min-1 was obtained, a value 30-fold lower than that of active MeHydA. Fe and S assays proved that only very little complex was incorporated into the protein, as confirmed by FTIR spectroscopy. Optimization of the procedure was achieved by varying the temperature, the incubation time, the excess of the chemical (based on the EasySpin package31) in MATLAB™ was carried out. Excess of the chemical was removed. These new maturation conditions were used to prepare active MeHydA for spectroscopic studies. The same protocol was used for reconstituting MeHydA with [Fe2(adt)(CO)4(CN)2]2–.

**Hydrogenase activity assay.** H2 production was determined according to a published procedure6 by using methyl viologen as an electron mediator and sodium dithionite as the reducing agent. Briefly, in all enzymatic assays as reported in Table S1 MeHydA (5-10 µL) corresponds to 0.2 nmol of hydrogenase was added to a total amount of 1.11 mL of 100 mM potassium phosphate, pH 6.8, 100 mM sodium dithionite, and 10 mM methyl viologen in a 10 mL vial sealed under anaerobic conditions (rubber stoppers, Carl Roth). Gas chromatograms were recorded on a GC System (Shimadzu GC-2014 with a thermal conductivity detector and a Quadrex column) and the amount of H2 was quantified using a calibration curve. [Fe2(adt)(CO)4(CN)2]2– and FeS-MeHydA were assayed as controls and did not show any detectable activity. All activities in this study were measured at least twice on 3 different protein preparations.

**Spectroscopic methods.** Pulse EPR spectra were recorded on a Bruker Elexys E580 spectrometer equipped with a dedicated Q-band super-QFT bridge. The 30 µL samples were accommodated in a homebuilt TE011 resonator. Cryogenic temperatures (10-20K) were reached using a custom made Helium closed cycle cryostat from Cryogenic Ltd. EPR signals were measured using FID detection following a 1µs excitation pulse.31 For the interpretation of all EPR experimental data, a home written simulation program (based on the EasySpin package31) in MATLAB™ was used.

FTIR measurements were performed on a BRUKER IFS 66 v/s FTIR spectrometer equipped with a Bruker MCT (mercury cadmium telluride) detector. The spectra were accumulated in the double-sided, forward-backward mode with 1000 scans (14 minutes) and a resolution of 2 cm-1 at 15 C. Data processing was facilitated by home written routines in the MATLAB™ programming environment. To obtain Hox-CO state sample was flushed with carbon monoxide for 20 minutes. Similarly, to isolate the Hox state the sample was flushed for 30 hours with argon gas. To access the reduced states the sample was flushed with hydrogen for few hours and treated with 10mM sodium dithionite buffer.

Mössbauer spectra were recorded on a conventional spectrometer with alternating constant acceleration of the γ-source. The sample temperature was maintained constant in an Oxford Instruments Variox cryostat. Isomer shifts are quoted relative to iron metal at 300 K. Mössbauer spectra were collected for frozen aqueous solution sample (2 mM, 650 µL) at 160 K and fitted using the program MFIT (written by Eckhard Bill, Max Planck Institute for Chemical Energy Conversion) with Lorentzian doublets.

**Electrochemistry.** All electrochemical experiments were carried out in a glove box (JACOMEX) filled with N2. The homemade pyrolytic graphite edge electrode was mounted onto a PINE MSR rotator. The potentiostat was model PGSTAT128N from METROHM. The buffer used in the electrochemistry experiments was a mixture of MES, CHES, HEPES, TAPS and Na- acetate, 5 mM each, plus NaCl 0.1M, titrated to pH 5, 7 or 9. In voltammetric experiments where the pH and H2 concentration were varied, intermediate pH values were obtained by mixing various amounts of these stock solutions, and partial pressures of H2 in the range 0.18-1 bar were obtained by bubbling in the electrochemical cell a mixture of H2 and Ar, the composition of which was adjusted using mass flow controllers (model SLA5800 from BROOKS Instruments). Circulating water in the double jacket of the electrochemical cell regulated the temperature. The open circuit potential (OCP) was measured before each experiment to make sure that the desired pH and partial pressure of H2 had been obtained; the reproducibility of the OCP between independent experiments was better than 4 mV. The contribution of the capacitive current was removed by averaging the forward and backward scans and offsetting the resulting signal so that the OCP was the same as that measured before recording the voltammogram.

The CO-inhibition electrochemical data were corrected for film loss32 and fitted with the program QSoas33 (www.qsoas.org), using the text file model "A<=I [kJ][co][ka]" and the command "QSoas> fit-kinetic-system model.txt /with=co:2,exp:common" where A and I stand for "active" (CO-free) and "inactive" (CO-bound), "t" is the number of successive injections of CO in the experiment, "common" means that the time constants for the exponential decay of [CO] after each injection are the same. "model.txt" is the text file that contains the kinetic model. Aerobic inactivation is studied in direct electrochemistry as for CO inhibition, although the time-dependent concentration of O2 is easily measured using a 2nd rotating electrode poised at low potential.13 This makes it easy to measure the time constant for the decay of the concentration of O2, which is used to constrain the fitting procedure.
Results

Preparation of MeH-HydA, the truncated form of HydA from M. elsdenii.

Figure S1 shows an alignment of the amino acid sequences of MeHydA and CpHydA. Both contain two ferredoxin-like clusters, each within a cysteine-rich CxxCxxxCxxxC motif, in the N-terminal domain. The significant degree of identity (40%) between the two proteins and the fact that CpHydA has been characterized by X-ray crystallography allowed the identification of potential sites for protein truncation, in order to remove the accessory clusters. The chosen truncated protein, named MeH-HydA, thus starts at residue V80 of MeHydA, corresponding to residue 208 in CpHydA between the β-strand 10 and α-helix 6. MeH-HydA has a theoretical molecular weight of 45.8 kDa and is designed to harbor the H-cluster exclusively. To facilitate purification a hexahistidine tag was added at the N-terminus. Plasmid preparation, expression and aerobic purification of apo-MeH-HydA are described in the supplementary information section (see Figure S2). Approximately 15 mg protein could be obtained from 1 L of culture. Gel filtration experiments showed that apo-MeH-HydA is a monomer in solution (Figure S2).

The purified protein contained negligible amounts of Fe (<0.2 ± 0.1 Fe per polypeptide chain) and thus was mainly in the apo-protein form. Its UV-visible light absorption spectrum displayed indeed only a very weak signal at 400 nm (Supplementary information, Figure S3a). One [4Fe-4S] cluster could be assembled upon incubation of the protein with an excess of iron ammonium sulfate and L-cysteine in the presence of dithiothreitol (DTT) and cysteine desulfurase (Supplementary information, Figure S3b and Figure S4a). The presence of a band at 400 nm (A400/A280 = 0.24) in the light absorption spectrum of the reconstituted protein (Supplementary information, Figure S4a) as well as iron and sulfur quantitation (Fe: 4.1 ± 0.2; S: 4.3 ± 0.2 per polypeptide) were consistent with the protein containing a single [4Fe-4S] cubane cluster. The cluster is EPR silent consistent with a S=0 [4Fe-4S]2+ state and excluding the presence of paramagnetic forms of the cluster. Upon anaerobic reduction with dithionite, it could be converted into an EPR active state. The rhombic signal (g = 2.043, 1.924, 1.892) is characteristic of a single S=1/2 [4Fe-4S]2+ cluster (Supplementary information Figure S4b). Spin quantitation, using Cu-EDTA as a standard, showed that about 40% of the clusters were reduced. Finally, the protein was characterized by Mössbauer spectroscopy after reconstitution of the cluster with 57Fe. The Mössbauer spectrum is shown in Figure S4c, Supplementary information. It can be simulated with a single pure [4Fe-4S]2+ cluster, containing two species (1:1) with parameters δ=0.31 mm/s ΔQ=0.97 mm/s and δ=0.50 mm/s ΔQ=0.98 mm/s.

These data clearly establish that, while lacking the whole N-terminal domain, MeH-HydA can properly assemble a redox-active [4Fe-4S] cluster.

Synthetic maturation of MeH-HydA: towards an active enzyme

To achieve full maturation of the protein we used the efficient chemical maturation protocol previously reported, which consists in the anaerobic treatment of the hydrogenase containing only the [4Fe-4S] cluster with an excess of a dinuclear iron complex, (Et4N)2[Fe2(adt)(CO)4(CN)2] (adt2- = azapropanedithiolate), mimicking the 2Fe-subcluster of HydA. In the supplementary information section we describe the exact MeH-HydA maturation protocol as it slightly differs from that previously used for MeHydA, in particular because lower pH values were required for optimized enzyme activation (Table S1). Maturation was monitored by assaying the protein for its Fe content and for hydrogenase activity using the standard methyl viologen/dithionite assay in phosphate buffer pH 6.8 as well as by FTIR spectroscopy.

Thus, when the reaction was carried out over 2 hours, in 0.1 M potassium phosphate buffer, pH 6, followed by desalting to remove the excess of complex, the resulting MeH-HydA enzyme displayed a large hydrogen evolution activity, 135 ± 15 µmol H2 mg-1 min-1 (Table S1), however lower than that of MeHydA (500 µmol H2 mg-1 min-1). Fe and sulfur quantitation showed that almost 50-60% of the H-cluster could be reconstituted under the best conditions. Once maturated, the protein is stable in anaerobic neutral aqueous solutions: no precipitation and no change in its light absorption spectrum could be observed after several hours at room temperature.

That the H-cluster was correctly assembled was further substantiated by spectroscopic characterization (Figure 1).

First, MeH-HydA was characterized by FTIR spectroscopy. The latter is indeed appropriate for identifying the CO (1800-2020 cm-1) and CN- (2040-2100 cm-1) vibrations associated with the CO and CN- ligands present in the 2Fe-subcluster. These signals can be clearly distinguished from those of the [Fe2(adt)(CO)4(CN)2]2- complex free in solution which are much broader. Moreover, it allows distinguishing between different H-cluster states usually coexisting in hydrogenase preparations. The FTIR spectrum of the protein after maturation (Figure 1A, MeH-HydA and Table S2) displayed sharp peaks corresponding to CN- and CO vibrations typical of those previously observed for MeHydA H-cluster in the Hox and Hox-CO state, in which an extra CO molecule binds the free coordination site on the 2Fe-subcluster. The excess of the synthetic complex used during maturation can be indeed a source of CO, which then reversibly binds to the 2Fe-subcluster, explaining why synthetic maturation usually results in a mixture of Hox and Hox-CO states. For unambiguous assignment of the FTIR peaks we treated the protein with an excess of CO to
generate a pure Hox-CO form (Figure 1, MeH-HydA CO). For reference, we also maturated MeH-HydA with the [Fe$_2$(pd$t=propanedithiolate)$(CO)4(CN)$_2$] complexes since, as previously reported for CrHydA$^{34}$, only Hox can be obtained in that case (Figure 1, MeH-HydA(pd$t$)). Extensive exposure to Ar gas converted active MeH-HydA into (mainly) Hox (Figure 1, MeH-HydA Ar). Specifically Hox-CO and Hox are well characterized by a peak at 2012 and 1945 cm$^{-1}$ respectively (to be compared with the spectrum of Hox-CO in MeH-HydA). In contrast, the spectrum of Hox-CO in MeH-HydA is only very slightly shifted with respect to the peak at 1896 cm$^{-1}$ (Figure 1, MeH-HydA Ar). The presence of a peak at 1944 cm$^{-1}$ indicates incomplete reduction. Table S2 compares the parameters obtained for MeH-HydA, MeHydA and CrHydA. Also the spectrum of as-maturated MeHydA is displayed in Figure S5 for comparison. It clearly shows that the active sites of MeHydA and CrHydA have very similar vibrational properties and furthermore that the truncation of the ferredoxin domain in MeHydA has almost no effect on these properties, demonstrating that the active site has remained intact.

Both Hox and Hox-CO states are paramagnetic and can be observed by EPR spectroscopy. In Hox, the 2Fe-subcluster is in the Fe(I)/Fe(II) (S=1/2) configuration while the [4Fe-4S] is oxidized (2+ state) and thus EPR silent. While its EPR signal is rhombic, that of Hox-CO is axial. The EPR spectrum of MeH-HydA (Figure 1B) clearly shows the presence of the two species, with Hox contribution decreasing upon addition of CO. The spectra have been simulated using parameters Hox: g$=(2.095, 2.039, 1.996)$; Hox-CO: g$=(2.0196, 2.009, 2.0079)$ (Figures 1B and S6). Figure S6 shows the Hox spectrum of Hox-HydA, Hox-CO, and then returns to its initial value, clearly demonstrating that the inhibition by CO is reversible. In contrast, and as observed with most HydAs (but not the enzyme from A. woodii$^{37}$, the inhibition by CO is partly irreversible at lower potentials (e.g. -360mV or -400 mV vs SHE, pH 7, Figure S7C); it has been argued that this is caused by the disruption of the bond between the [4Fe-4S] and 2Fe-subcluster.$^{36,38}$

The rate constant of CO binding and release were determined by fitting a model that assumes (pseudo)first order kinetics in both directions.

$$\frac{k_{\text{on}}^{\text{CO}}}{k_{\text{off}}^{\text{CO}}} = \frac{[\text{CO}]}{1} \quad (1)$$

The obtained rate constant values for both forms of the enzyme are shown in Table S3, together with previously published values obtained for CaHydA, CrHydA, DdHydAB and Acetobacterium woodii HydA. Analysis of the $k_{\text{on}}^{\text{CO}}$ values clearly shows that inhibition by CO is slower in the case of MeHydA and MeH-HydA than with any previously investigated HydA.

### Reaction with dioxygen

The same approach was used for characterizing the inhibition by O$_2$ quantitatively, using the 4 enzymes MeHydA, MeH-HydA, CaHydA and CrHydA. Figure 2A shows the current against time trace (in black) obtained upon exposure of MeHydA to O$_2$, and that (in blue) obtained with CaHydA. In both cases, like for other HydAs studied before, the reaction can only be described by a two-step mechanism$^{13,37,39}$, which assumes that the active form of the enzyme (A) reacts with O$_2$ during a bimolecular step to form an O$_2$ adduct (I), which can either be repaired or be irreversibly transformed into a dead-end species (D):

$$\frac{k_{\text{on}}^{O_2}}{k_{\text{off}}^{O_2}} \times [O_2] = \frac{k_3}{k_2} \quad (2)$$
Figure 1: FTIR and EPR spectra of MeH-HydA. A) From bottom to top: FTIR spectrum of MeH-HydA maturated with [Fe2(adt)(CO)4(CN)2]2– (Hox+Hox-CO); maturated with [Fe2(pdt)(CO)4(CN)2]2– (Hox); maturated with [Fe2(adt)(CO)4(CN)2]2– and flushed with Ar (mainly Hox); maturated with [Fe2(adt)(CO)4(CN)2]2– and treated with CO (Hox-CO); maturated with [Fe2(adt)(CO)4(CN)2]2– and treated with H2 100% and 10 mM Na-dithionite (DT) (Hred, HredH+, Hox). B) Q-band FID-detected EPR spectra measured at 20K for active MeH-HydA before and after treatment with CO and their simulations. Information about signal positions is indicated and assigned to different redox states by color code: red indicates Hox, light blue Hox-CO, green, pink and blue reduced states; and black overlapping signals.

With MeHydA we obtained the following values: $k_{inO2}^2=0.25\pm0.1$ mM$^{-1}$s$^{-1}$, $k_3=0.05\pm0.02$ s$^{-1}$, $k_4=0.015\pm0.007$ s$^{-1}$ and thus $k_{eff}=k_{inO2}^2k_3/(k_4+k_3)=0.075$ mM$^{-1}$s$^{-1}$, $k_{eff}$ being the “global” second-order rate of irreversible formation of the inactive forms, defining the “overall” sensitivity to O2. The rate constants collected in Table 1 clearly show that MeHydA reacts with O2 in the first step ($k_{inO2}^2$ values) much more slowly than any other HydA studied so far; the inset of Fig 2A clearly shows a difference in initial rates of reaction with O2 of MeHydA and CaHydA. This nicely parallels the observed slow reaction of MeHydA with CO. A typical experiment showing the inhibition by O2 of MeH-HydA is shown as a black trace in Fig 2B.

Table 1. Kinetic parameters for the reaction of HydA enzymes with O2

<table>
<thead>
<tr>
<th></th>
<th>$k_{inO2}$</th>
<th>$k_a$</th>
<th>$k_3$</th>
<th>$k_{eff}=k_{inO2}^2k_3/(k_4+k_3)$</th>
<th>T</th>
<th>E</th>
<th>References</th>
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</thead>
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<tr>
<td>MeHydA</td>
<td>0.25±0.1</td>
<td>0.05±0.02</td>
<td>0.015±0.007</td>
<td>0.075</td>
<td>20</td>
<td>+40</td>
<td>This work</td>
</tr>
<tr>
<td>MeH-HydA</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CaHydA</td>
<td>0.9±0.2</td>
<td>0.07±0.02</td>
<td>0.004±0.00</td>
<td>0.05</td>
<td>20</td>
<td>+40</td>
<td>This work</td>
</tr>
<tr>
<td>CrHydA</td>
<td>2.5</td>
<td>0.035</td>
<td>0.024</td>
<td>1.02</td>
<td>12</td>
<td>+40</td>
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<tr>
<td>A. woodii HydA</td>
<td>6.5</td>
<td>0.005</td>
<td>0.0032</td>
<td>2.5</td>
<td>30</td>
<td>+40</td>
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<tr>
<td>DdHydAB</td>
<td>40</td>
<td>0.15</td>
<td>n/d</td>
<td>n/d</td>
<td>30</td>
<td>+200</td>
<td></td>
</tr>
</tbody>
</table>

n/a: not applicable; n/d: not determined
Catalysis

The catalytic responses (steady-state voltammograms) of MeHydA and MeH-HydA are shown in panels A and B of Figure 3. At the slow scan rates used here, the response is in a steady-state regime (it is independent of scan rate). The shapes of the signals are significantly different; in particular MeH-HydA appears to be more biased in the direction of H⁺ reduction (the current is larger in the reductive direction). (Note that the bias relates to the magnitude of the current in each direction; the direction of the reaction and thus the sign of the current are defined by the sign of the difference between the electrode potential and the open circuit potential). Preliminary study of a truncated form of CaHydA also showed a comparable change of the voltammograms. Here we apply previous models to make sense of this change in electrochemical signature. We previously described the kinetic models that can be fitted to these data. The simplest model, called EEC, reduces the enzyme to a two-electron active site that can directly exchange electrons with an electrode. A more complex model, EEC(R), explicitly considers a one-electron relay that mediates electron transfer between the electrode and the active site (see Figure S8 for details). Regarding MeHydA, Figures 3A and C show that the EEC(R) model fits the data much better than the EEC model (the derivatives of the data are shown in panels C and D, because simultaneously fitting a model to the data and to the derivative of the data greatly increases the reliability of the fit). In contrast, for MeH-HydA both models are equivalent. This is as expected considering the cofactor content in these two enzymes, meaning that the fitting procedure can tell which of the two enzymes has accessory clusters. We have already reached this conclusion in a previous comparison of CaHydA and CrHydA. The examination of the best parameters of the fits in Table S4 tells us why MeHydA is better (relatively) at oxidizing H₂ than MeH-HydA. First, for both enzymes, the fits (of the two distinct models) converge on the same values of the two one-electron potentials of the active site (\(E^0_1 \approx -410 \text{ mV vs SHE}\) and \(E^0_2 \approx -475 \text{ mV vs SHE}\)) and the “intrinsic” value of the bias (\(k_2/k_{-2} \approx 15\) in the reductive direction). This is consistent with the conclusion from spectroscopy that the truncation does not affect the active site. Second, for MeHydA, the fit identifies a very slow electron transfer step in both directions (\(k_1\) and \(k_{-1}\)), between the relay and the most oxidized active site redox couple (O/I). This step determines the rate of turnover in both directions, so that the catalytic bias of MeHydA is not determined by the ratio \(k_2/k_{-2}\), but rather by the ratio \(k_{-1}/k_1\). Since this ratio equates \(\exp(\text{RT} (E^0_{11} - E^0_{R}))\), it means that, in the particular case of MeHydA, the potential difference between the relay and one of the two active site one-electron potential determines the catalytic bias of the wild type enzyme.
To determine whether the above conclusion holds over a range of experimental conditions, we performed and interpreted a series of experiments that consisted in recording the steady-state voltammetric responses of both MeHydA and MeH-HydA at pH values from 6.5 to 8.5 and H₂ partial pressures in the range 0.18 to 1 bar. For each set of experimental conditions the EEC model was fitted to the voltammetric data of the MeH-HydA enzyme to determine the two active site redox potentials, and these values were used to constrain the fit of the EEC(R) model to the MeHydA voltammograms recorded under the same conditions, to obtain the values of $E^{0}_1$, $E^{0}_2$, and $E^{0\text{R}}$. The fits are all shown in SI Figures S9 and S10. Figure 4A shows the values of $E^{0}_1$, $E^{0}_2$, and $E^{0\text{R}}$ obtained at different H₂ concentrations against pH, and panel B shows the values of $k_2/k_2$. Dark symbols correspond to data recorded 100% H₂, lighter symbols show the values obtained from the data recorded under 55%, 35% and 18% H₂; a small offset along the X-axis decreases the overlap between the data points recorded at the same pH. We observed the following features. First, the catalytic bias of the WT enzyme in favor of H₂ reduction, defined as the ratio of the limiting currents in the two directions of the reactions (and determined from the

Figure 3: Catalytic signals (H₂ oxidation at high potential, and production at low potential) for MeHydA and MeH-HydA. MeHydA (left) and MeH-HydA (right) are covalently attached to a rotating disc graphite electrode (plain black lines). T = 20°C, pH 7, 1 bar H₂, ω = 3 krpm, 20 mV/s. Panels A and B show the steady-state voltammograms (after correction for the capacitive current) and panels C and D the first derivative of the data. The dashed red and blue lines are the best fits using the EEC and EEC(R) models, respectively, defined in reference,40,42 The small panels show the residues for each fit. The values of the optimized parameters are listed in Supporting Information, Table S4.
extrapolation of the current calculated with the EEC(R) model) increases when the H₂ concentration is raised and when the pH is decreased. This is as observed before for various NiFe and FeFe hydrogenases including MeHydA. The following analysis clarifies why this occurs in the case for MeHydA and shows that this is not merely due to substrate availability. Second, the value of \( k_2/k_2 \) (Fig. 4B) increases in proportion to H⁺ concentration (one hundred fold over 2 pH units) and decreases when [H₂]; this pH dependence is not unexpected considering that \( k_2 \) includes H₂ binding, and it suggests that \( k_2 \) includes protonation (this is consistent with the observation that \( E_0^2 \) is independent of pH, meaning that not all intramolecular electron transfer steps are coupled to protonation, hence one proton must be taken up after the full oxidation of the active site). Below we show that \( k_2/k_2 \) is not the determinant of the bias. Third, the value of \( E_0^1 \) (green in Fig. 4A) decreases exactly 60mV per pH unit (green dotted line) as expected for a one-electron one-proton reaction. This is true at all H₂ concentrations. Fourth, the values of \( E_0^2 \) is less pH-dependent (-20mV/pH, blue dotted line) and independent of [H₂]. Fifth, the value of \( E_0^k \) shows little dependence on pH under 100% H₂ (-24mV/pH), and a slightly larger pH dependence at lower H₂ concentrations (up to -30mV/pH under 18% H₂, still much less than the -60mV/pH expected for a fully coupled electron/proton transfer; the small pH dependence may be due to the fits of the data recorded under low [H₂] being less reliable). A small dependence of the potential on pH is what we expect for a FeS cluster involved in electron transfer, and we consider that this observation validates the theoretical models and methods used here. We note that the pH 8 value of \( E_0^k = -440 \text{ mV} \) is not far from the value of -370 mV obtained from EPR titrations for the FS4A and FS4B clusters of Cpl hydrogenase.

Fig. 5 shows the relation between the catalytic bias, \( k_2/k_2 \) (circles) and \( k_1/k_1 \) (squares), for all data. The dashed red line shows \( y=x \), and matches the values of \( k_1/k_1 \), showing that the latter determines the bias. This confirms the above conclusion that the catalytic bias of the WT enzyme is determined by the kinetics of intramolecular electron transfer, and this is so over a large range of experimental conditions.

**Discussion**

We have thus been able to engineer the [FeFe]-hydrogenase from *M. elsdenii*, MeHydA, to generate a significantly shorter variant, MeH-HydA, which lacks the N-terminal ferredoxin domain. Even though the deletion accounts for only 15% of the protein, it is the first attempt to go in this direction. More extensive deletion might be considered in the future. The protein expresses and folds well and is highly active after maturation. As shown by FTIR, EPR and Mössbauer spectroscopic characterization, the H-cluster correctly assembles in MeH-HydA, with spectroscopic properties almost identical to those of MeHydA and CrHydA. The only remarkable difference distinguishing MeH-HydA from the other [FeFe]-hydrogenases is the deviating EPR spectrum of the Hox-CO state (Figures 2b and S6) which is less anisotropic.

![Figure 4: The parameters obtained from fitting the EEC and EEC(R) models to steady-state voltammograms obtained with MeH-HydA and MeHydA in a range of pH (6.5 to 8.5) and H₂ concentration (from 10% to 100%). The potentials of the active site and of the relay (panel A) and the "intrinsic" bias of the active site, \( k_2/k_2 \), as defined in Fig. S8 and in ref 44. See Figs S9 and S10. Dark symbols correspond to values obtained from the data recorded under 100% H₂, and lighter symbols indicate H₂ partial pressures of 0.55, 0.3 and 0.18 bar. T=20°C, \( \omega = 3 \text{ krpm}, 20 \text{ mV/s} \). Data obtained at different H₂ concentrations are slightly offset along the pH axis for clarity.](image1)

![Figure 5: Evidence that the catalytic bias (the ratio of limiting currents) is determined by \( k_1/k_1 \) over a large range of pH values and [H₂] concentrations. Fitted values of \( k_2/k_2 \) (circles) or \( k_1/k_1 \) (squares) (\( k_2 \) and \( k_1 \) are both in the direction of H₂ evolution in the scheme of Fig. S8) plotted against the experimental value of the catalytic bias (\( i_{\text{red}}/i_{\text{ox}} \)) for different values of the pH and of the partial pressure of H₂ (from 100% dark symbols to 18%, light grey symbols). Data obtained in the pH range 6.5 (rightmost) to 8.5 (leftmost) from experiments using](image2)
zyme and CO binding at the active site. 17,40 Since the kinetics of both long-range CO diffusion within the enzyme is at least 10-fold lower than in any other HydA (Table 1). Considering the above conclusion that the rate of CO binding/release depends on the rate of CO binding was up to 40-fold and 4-fold lower than that obtained in the case of CrHydA and CaHydA respectively and it was even 500-fold lower than in the case of DdHydAB (Table S3). The rate of CO binding/release depends on the intramolecular diffusion rates. Which amino-acids are responsible for making intramolecular diffusion so slow in MeHydA is now being investigated further in our groups.

Second, the reaction of MeHydA with O2 appears to follow the same mechanism as that previously described for CaHydA and CrHydA 15,17 as well as for Acetobacter woodii HydA. 37 The value of the rate constant k inO2, which depends on O2 intramolecular diffusion through the protein and binding at the distal Fe of the H-cluster17, is at least 10-fold lower than in any other HydA (Table 1). Considering the above conclusion that the intramolecular diffusion of CO is particularly slow in MeHydA, it is reasonable to assume that this is also the reason why O2 inhibition is slow. However, the initial O2 binding step is not the parameter that strictly defines the “overall” sensitivity to O2, because what matters is the rate of irreversible formation of the inactive forms, which is the effective 2nd-order rate defined by k eff = k inO2 / (k a + k 3) whose calculated values are shown in Table 1. 42 The k eff value is about the same for MeHydA and CaHydA despite the fact that the individual rate constants are significantly different. In particular, the smaller value of k inO2 in MeHydA is compensated for by the smaller value of k a and the larger value of k 3. According to the model in ref 17, these two rate constants correspond to the complex redox chemistry of the O2 bound adduct and have no simple meaning. They can be affected by electron availability as well as the kinetics of proton transfer in the enzyme.

We show here that MeH-HydA and MeHydA display comparable reactivities with respect to the gaseous inhibitors CO and O2. A recent related report using a truncated form of CaHydA describes similar observations, even though this work suffers from incomplete characterization of the truncated form of CaHydA. 23 This together with the observation that CrHydA, lacking accessory clusters, and DdHydAB, containing two ferredoxin-type clusters, are both very sensitive to CO and O2, show that previous suggestions regarding the protective role of the accessory clusters from O2 might not be necessarily correct in the case of [FeFe]-hydrogenases. 17 This obviously indicates that understanding O2 sensitivity of HydAs remains challenging. Nevertheless, MeH-HydA is much more O2-resistant than CrHydA (Figure 2 and Table 1), thus showing that it is possible to engineer a complex HydA enzyme to generate a simple, active and significantly O2-resistant CrHydA-like enzyme, which was one of the objectives of this work.

Figure 3 clearly shows that both MeHydA and MeH-HydA forms are biased in the direction of H2 production (the catalytic currents are larger in the direction of reductive catalysis), but unexpectedly MeH-HydA much more so than MeHydA. Since the active site is not affected by the truncation, a major role of the iron-sulfur electron transfer chain of MeHydA in tuning the enzyme’s catalytic bias is inferred. Comparing the results of the fits for the two versions of the same enzyme (with and without the relays) tells us why the catalytic bias is changed. In MeH-HydA, the catalytic bias is simply k in / k out = k 2 / k -2 = 15 at pH 7, 100% H2 (Figures 3 and S8), with k 2 (in the direction of H2 evolution) and k -2 being the rate constants that define the chemical (as opposed to redox) steps in the catalytic cycle. The ratio k 2 / k -2 is therefore a property of the active site. Our data show that this ratio is about the same for MeH-HydA and for MeHydA, again in agreement with the truncation having no significant effect on the active site. However, in the case of MeHydA, the intramolecular electron transfer step (between the relay and the active site, k 1 and k -1 in Figure S8) is slow and fully determines the catalytic rates in both directions. As a consequence, the catalytic bias for MeHydA is instead given by the ratio k 1 / k -1, which is determined by the difference between the potential of the relay and that of a particular one-electron redox reaction at the active site. Here, i red / i ox = k 1 / k -1 = exp(F/RT(E01 - E0R)) ≃ 2 at pH 7, 100% H2, with E01 being the potential of one of the two redox transitions of the active site and E0R the potential of the relay. (Note that the equation above is not the same as eq. 4 in ref 50, which tentatively links the bias to the difference between E0R and the open circuit potential.) The slow intramolecular ET step compensates for the strong “intrinsic” preference of the active site to catalyse H2 production over H2 oxidation, and entirely defines the catalytic bias. We have shown (see the discussion of Figs 4 and 5) that this is true over a large range of pH and [H2].

In that regard, it is interesting to parallel our observations to a recent analysis of two HydAs, named Cpl and Cpl II, within C. pasteurianum. 51 Cpl has extremely high hydrogen production activity in comparison to Cpl II, while Cpl II has elevated hydrogen oxidation activity in comparison to Cpl, thus with biases in tight relation with their respective physiological functions. How the bias is tuned in these enzymes is unclear but it is tempt-
ing to relate it, at least in part, to the established absence of a ferredoxin domain and accessory factors in CpII, like in MeH-HydA, which are present in CpI. This finally further illustrates the notion, previously raised in the case of [NiFe]-hydrogenases, that the bias may not be mainly determined by redox properties of the active site but rather by reaction steps occurring on sites that are remote from the active site. However, the mechanism that selects the catalytic bias is unprecedented: in NiFe hydrogenases, the rate limiting step is not the same when the enzyme evolves or oxidizes H₂, and site-directed mutations that block the gas channel or slow intermolecular electron transfer selectively slow the rate of the step that limits H₂ evolution or H₂ oxidation, respectively. Regarding MeHydA the data are consistent with intramolecular electron transfer being rate limiting in both directions of the reactions over a range of experimental conditions, and the rate of this particular step determining the catalytic bias (Fig. 5).

This is certainly a new research direction that can be further addressed using the interesting pair of enzymes MeHydA and MeH-HydA.

Conclusion

All these data show that it is possible to engineer HydAs to generate active hydrogenases that combine the resistance of the most resistant HydAs and the simplicity of algal HydAs, containing only the H-cluster. They also clearly establish that the accessory clusters, at least in MeHydA, are key molecular components for defining the catalytic bias due to the slow and limiting intramolecular electron transfers. In contrast, at least when the enzyme is attached to an electrode surface, they play no role in controlling the sensitivity to O₂. Whether this can be generalized to other HydAs remains to be shown. These engineered enzymes thus provide unique tools to further study basic issues, such as the tunability of their catalytic bias.

ASSOCIATED CONTENT

Supporting Information. Sequence alignment of CpI with MeHydA and MeH-HydA, purification profiles of apo- and Fe₅-S-MeH-HydA, EPR, Mössbauer spectra, chronoamperometry of CO inhibition and additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGEMENTS

The Fondation de l’Orangerie for individual Philanthropy, the French State Program ‘Investissements d’Avenir (Grants “LABEX DYNAMO”, ANR-11-LABX-0011, and “LABEX ARCANE”, ANR-11-LABX-0003-01) supported this work. The authors thank Pr. Philippe Soucaille for his contribution to this work through helpful discussions and JW Peters for providing CrHydA-expressing plasmid. The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University - A*MIDEX, a French “Investissements d’Avenir” programme. The French authors are part of the French bioinorganic chemistry research network (www.frenchbic.cnrs.fr).

References


Supplementary Information

Engineering an [FeFe]-hydrogenase:
do accessory clusters influence O₂ resistance and catalytic bias?

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Figures

Figure S1: Sequence alignment of CpHydA, MeHydA and MeH-HydA proteins.

Figure S2: Apo-MeH-HydA purification.

Figure S3: [Fe–S] reconstitution of apo-MeH-HydA.

Figure S4: Spectroscopic properties of reconstituted MeH-HydA.

Figure S5: FTIR spectrum of MeHydA

Figure S6: Comparison of simulated EPR spectra of MeH-HydA and MeHydA.

Figure S7: Reversible inhibition by CO.

Figure S8: Kinetic schemes

Figure S9: The fits of the EEC(R) model to data recorded with MeHydA in the range of pH 6.5 to 8.5 and H2 partial pressure 18 to 100%.

Figure S10: The fits of the EEC model to data recorded with MeH-HydA (respectively) in the range of pH 6.5 to 8.5 and H2 partial pressure 18 to 100%.

Table S1: Hydrogenase specific activity

Table S2: FTIR frequencies for MeH-HydA, MeHydA and CrHydA1.

Table S3: Kinetic parameters for the inhibition of various HydAs by CO.

Table S4: Best parameters obtained for the fits of cyclic voltammograms in Fig.3.
Figure S1: Sequence alignment of CpHydA, MeHydA and MeH-HydA proteins using clustalo\textsuperscript{1}. Secondary structures are calculated using the crystal structure of CpHydA (pdb 3c8y). The cysteine residues chelating the [4Fe-4S]\textsubscript{4} cluster are highlighted in yellow with the cysteine linking the 2Fe-subcluster marked with a star; conserved residues are highlighted in red. Cysteines boxed in cyan coordinate the [2Fe-2S] cluster in CpHydA. Residues boxed in orange, green and magenta coordinate the auxiliary [4Fe-4S] clusters. The figure was generated with ESPript3.\textsuperscript{2}
Figure S2: Apo-MeH-HydA purification. Apo-MeH-HydA was purified to homogeneity in two chromatographic steps (a and b). a) Ni-NTA chromatography elution profile using an imidazole gradient. b) Size exclusion chromatography elution profile on a Superdex S200 16/600 in 50 mM Tris-HCl buffer pH 8.0, 200 mM NaCl, 5 mM DTT. c) Anaerobic size exclusion chromatography elution profile on an analytical Superdex S200 10/300 in 50 mM Tris-HCl pH 8.0 containing 300 mM NaCl, 10% glycerol and 5 mM DTT. The elution volume of the apo-MeH-HydA is 14.55 mL. The inset displays a SDS gel after chromatography. d) Superdex S200 10/300 GL calibration curve using Thyroglobulin (670 kDa, 9.22 mL), γ-globulin (158 kDa, 12.32 mL), Ovalbumin (44 kDa, 15.2 mL) and Myoglobin (17 kDa, 17.03 mL). The derived molecular weight suggests that apo-MeH-HydA is monomeric.
Figure S3: [Fe–S] reconstitution of apo-MeH-HydA. a) UV-visible spectrum of 25 µM apo-MeH-HydA in 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM DTT in a 1 cm pathlength cuvette. b) UV-visible absorption spectra of 100 µM MeH-HydA recorded every 20 minutes during [Fe–S] cluster reconstitution. Reaction mixture (100 µM apo MeH-HydA in the presence of 5 molar excess of Fe (II) and L-cysteine, 10 mM DTT and a catalytic amount of cysteine desulfurase) in 50 mM Tris pH 8, 300 mM NaCl, 10% v/v glycerol using a 1 mm pathlength cuvette. c) S200 elution profile of apo-MeH-HydA (red) and FeS-MeH-HydA (dashed black).
Figure S4: Spectroscopic properties of [FeS]-reconstituted MeH-HydA. a) UV-visible spectra of apo and [FeS]-
reconstituted protein b) Q-band FID-detected EPR spectra measured at 10K of reduced (10mM NaDT) protein

together with simulation. G-values are given at the top of figure. c) Mössbauer spectrum of [\textsuperscript{57}FeS]-reconstituted
MeH-HydA measured at 160K in zero magnetic field. Spectrum was simulated with two components with equal
contribution. Parameters given in the figure are in mm/s. The grey horizontal bars represent the residual of the fit
shifted into the vertical range of the experimental Mössbauer spectra.
Figure S5: FTIR spectra of MeH-HydA (red) and MeHydA (black) in the Hox state. The feature at 2013 cm$^{-1}$ (*) shows the presence of small amounts of Hox-CO state in MeHydA.
Figure S6: Comparison of simulations of EPR spectra obtained for MeHydA and MeH-HydA in H_{ox} and H_{ox-CO} states. g-values used for simulations are given above the spectra.

For MeH-HydA in the H_{ox} state a slight shift of the g-values (up to 0.008) is observed with respect to MeHydA. This shift can be explained by slightly different geometry of the H-cluster. In the spectrum of the H_{ox-CO} state of MeH-HydA all the lines are collapsing creating a very narrow spectrum which was not observed for any of the other known hydrogenase species. However, the relaxation properties are similar to those of other hydrogenases. This unusual behaviour cannot be explained at the moment considering the fact that the FTIR spectrum obtained for this redox state is identical to those of other [FeFe]-hydrogenases.
Figure S7: Reversible inhibition by CO. Panel A: reaction of MeHydA with CO at pH 7, E = -160 mV vs SHE, 20°C. The black line is the current normalized by its value before CO is added, corrected for film loss. The red line is the fit to the model in ref \(^3\), used to measure the kinetic constants for CO binding and release. Panel B: same experiment as in Panel A but carried out with MeH-HydA. Panel C: reaction of MeHydA with CO at pH 7, 1 bar H\(_2\), 20°C, showing that the reaction with CO is less reversible at lower potential (note that all potentials used here are above the open circuit potential; the lower the potential the lower the H\(_2\)-oxidation catalytic current). The electrode potential was stepped as indicated by the red dashed line and the right axis.
The letter A with a subscript O (for oxidized”), I (intermediate), or R (reduced) depicts the redox state of the active site. In the rightmost scheme, “R” is the relay that can be either oxidized or reduced. Each redox step (“E”) might be coupled to reversible chemical reactions (not depicted), which are supposed to be very fast on the time scale of turnover, and therefore at equilibrium. This may include (de)protonations and/or the binding and release of the substrate/product. Such fast-coupled reactions affect the apparent values of the reduction potentials and the apparent rates of interfacial ET. The catalytic cycles are closed by reversible chemical reactions (“C”), whose first-order (or pseudo-first-order) rates $k_{\pm 2}$ are allowed to be slow on the time scale of turnover. For these reactions, positive subscripts correspond to the catalytic cycle turning in the direction that reduces the substrate (e.g., H$_2$ evolution in the case of hydrogenases). The pH and substrate/product concentrations are not explicitly considered, but, of course, they may affect the parameters of the model.

**Figure S8:** The kinetic schemes used here and described in refs $^4, ^5$

The schemes and the following text are copied from $^4$ (© 2013 American chemical society, with permission).
Figure S9: The fits of the EEC(R) model to data recorded with MeHydA in the range of pH 6.5 to 8.5 and H₂ partial pressure 18 (blue) to 100% (red). Data are shown as continuous lines, and the best fits as dotted lines. The best parameters are shown in main text Figures 4 and 5.
Figure S10: The fits of the EEC model to data recorded with MeH-HydA in the range of pH 6.5 to 8.5 and H₂ partial pressure 18 (blue) to 100% (red). Data are shown as continuous lines, and the best fits as dotted lines. The best parameters are shown in main text Figures 4 and 5.
Table S1: Specific hydrogenase activity of MeH-HydA depending on different maturation conditions.

<table>
<thead>
<tr>
<th>pH of maturation</th>
<th>pH activity</th>
<th>T (°C)</th>
<th>Specific activity (µmol H₂·mg⁻¹·min⁻¹)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0**</td>
<td>8.0</td>
<td>21</td>
<td>N.A.</td>
<td>2</td>
</tr>
<tr>
<td>6.8</td>
<td>6.8</td>
<td>21</td>
<td>23 ± 3.5</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>21</td>
<td>N.F</td>
<td>16-24</td>
</tr>
<tr>
<td>6.8*</td>
<td>6.8</td>
<td>21</td>
<td>20 ± 2.0*</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>6.8</td>
<td>21</td>
<td>2.2 ± 0.3</td>
<td>16-24</td>
</tr>
<tr>
<td>6.8</td>
<td>6.8</td>
<td>21</td>
<td>17.5 ± 2.5</td>
<td>16-24</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>37</td>
<td>N.A.</td>
<td>16-24</td>
</tr>
<tr>
<td>6.8</td>
<td>6.8</td>
<td>37</td>
<td>3.2 ± 0.8</td>
<td>16-24</td>
</tr>
<tr>
<td>6.8</td>
<td>6.8</td>
<td>37</td>
<td>2.5 ± 0.5</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>6.8</td>
<td>21</td>
<td>125 ± 15</td>
<td>2</td>
</tr>
<tr>
<td>6***</td>
<td>6.8</td>
<td>21</td>
<td>80 ± 10</td>
<td>2</td>
</tr>
<tr>
<td>6****</td>
<td>6.8</td>
<td>21</td>
<td>135 ± 10</td>
<td>2</td>
</tr>
<tr>
<td>5.5*****</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>5*****</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

The data shown are mean values ± s.d. * Maturation has been carried out with 10 molar excess of the hybrid 1HydF in the same buffer conditions. ** N.A.=No activity *** The excess of [Fe₂(adt)(CO)₄(CN)₂]²⁻ was increased to 100-fold with respect to protein concentration. In all the other tests the complex was used in a 10-fold excess with respect to protein concentration. **** In bold the best conditions obtained after removing the excess of the chemical. ***** Protein precipitated.
**Table S2.** FTIR frequencies (in cm$^{-1}$) corresponding to the CN$^-$ and CO stretching vibrations of CrHydA, MeHydA and MeH-HydA maturated with [Fe$_2$(adt)(CO)$_4$(CN)$_2$]$^2^-$. In bold, the most characteristic and prominent signals for each redox state.

<table>
<thead>
<tr>
<th>state</th>
<th>ν(M-CN$^-$)</th>
<th>ν(M-CO)</th>
<th>ν(M-CO-M)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_{ox}$-CO</td>
<td>2090</td>
<td>2013, 1969, 1955</td>
<td>1804</td>
<td>6</td>
</tr>
<tr>
<td>H$_{ox}$</td>
<td>2087, 2079</td>
<td>1964, 1937</td>
<td>1803</td>
<td>6</td>
</tr>
<tr>
<td>H$_{red}$</td>
<td>2069, 2041</td>
<td>1956, 1916, 1891</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>H$_{ox}$-CO</td>
<td>2095, 2089</td>
<td>2012, 1970, 1965</td>
<td>1804</td>
<td>this work</td>
</tr>
<tr>
<td>H$_{ox}$</td>
<td>2089, 2079</td>
<td>1967, 1943</td>
<td>1801</td>
<td>this work</td>
</tr>
<tr>
<td>H$<em>{red}$/H$</em>{red}$H$^+$*</td>
<td>-</td>
<td>1985, 1955, 1937, 1896, 1891</td>
<td>-</td>
<td>this work</td>
</tr>
<tr>
<td>H$_{ox}$-CO</td>
<td>2092, 2084</td>
<td>2013, 1970, 1964</td>
<td>1810</td>
<td>7</td>
</tr>
<tr>
<td>H$_{red}$/CO</td>
<td>2086, 2075</td>
<td>2002, 1967, 1951</td>
<td>1793</td>
<td>7</td>
</tr>
<tr>
<td>H$_{ox}$</td>
<td>2088, 2072</td>
<td>1963, 1939</td>
<td>1803</td>
<td>8</td>
</tr>
<tr>
<td>H$_{red}$</td>
<td>2083, 2067</td>
<td>1962, 1933</td>
<td>1791</td>
<td>8</td>
</tr>
<tr>
<td>H$_{red}$H$^+$</td>
<td>2071, 2032</td>
<td>1968, 1914, 1891</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>H$_{red}$H$^+$</td>
<td>2067, 2027</td>
<td>1953, 1917, 1881</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>H$_{red}$H$^+$</td>
<td>2082, 2068</td>
<td>1978, 1960</td>
<td>1860</td>
<td>9</td>
</tr>
</tbody>
</table>

* While some features allow to distinguish the two reduced states (1937 cm$^{-1}$ for H$_{red}$ and 1896 cm$^{-1}$ for H$_{red}$H$^+$), no clear assignment of the other features can be done at this time.

As far as MeH-HydA is concerned, in the IR traces of Figure 1 one can distinguish the two reduced states (1937 cm$^{-1}$ for H$_{red}$ and 1896 cm$^{-1}$ for H$_{red}$H$^+$). It is not clear if the doubly reduced state (H$_{red}$H$^+$) is present. However, the two overlapping signals at 1896 and 1891 cm$^{-1}$ could correspond to the main signals of both H$_{red}$H$^+$ and H$_{red}$H$^+$. Therefore, there is no possibility for a clear assignment at this point.
Table S3. Kinetic parameters for the inhibition of various HydAs by CO at pH 7.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{in}^{CO}$ (a)</th>
<th>$k_{out}^{CO}$</th>
<th>T</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM$^{-1}$s$^{-1}$</td>
<td>s$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeHydA</td>
<td>2 (a)</td>
<td>0.003</td>
<td>20°C</td>
<td>This work</td>
</tr>
<tr>
<td>MeH-HydA</td>
<td>4 (a)</td>
<td>0.005</td>
<td>20°C</td>
<td>This work</td>
</tr>
<tr>
<td>CaHydA</td>
<td>8 (a)</td>
<td>0.03</td>
<td>30°C</td>
<td>10,11</td>
</tr>
<tr>
<td>CrHydA</td>
<td>80 (a)</td>
<td>0.015</td>
<td>30°C</td>
<td>10</td>
</tr>
<tr>
<td>A. woodii HydA</td>
<td>200 (a)</td>
<td>0.02</td>
<td>30°C</td>
<td>12</td>
</tr>
<tr>
<td>DdHydAB</td>
<td>1000 (b)</td>
<td>0.03</td>
<td>30°C</td>
<td>10</td>
</tr>
</tbody>
</table>

(a) Raw values under 1 atm de H$_2$, not corrected for the small protective effect of H$_2$ (b) corrected for the protection by H$_2$. Note that since the $K_m$ is large and similar for all these enzymes, the correction is small. $k_{in}^{app} = k_{in}^{CO}(no H_2) / (1+[H_2]/K_m)$. 


Table S4. The best parameters of the fits shown in Figure 3, pH 7 100% H₂, as defined in ref sup3 and Figure S8.

<table>
<thead>
<tr>
<th>MeH-HydA in Figs 4B&amp;D, EEC model</th>
<th>MeHydA in Figs 4A&amp;C, EEC(R) model</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E^0_1$ (mV vs SHE)</td>
<td>$E^0_1$ (mV vs SHE) (*)</td>
</tr>
<tr>
<td>$E^0_2$ (mV vs SHE)</td>
<td>$E^0_2$ (mV vs SHE) (*)</td>
</tr>
<tr>
<td>$E^0_R$ (mV vs SHE)</td>
<td></td>
</tr>
<tr>
<td>$k_2/k_{-2}$</td>
<td>$k_2/k_{-2}$</td>
</tr>
<tr>
<td>$k_1/k_2$</td>
<td>$k_1/k_2$</td>
</tr>
<tr>
<td>$k'<em>{1}/k</em>{2}$</td>
<td>$k'<em>{1}/k</em>{2}$</td>
</tr>
<tr>
<td>$k^{0}<em>{1}/k^{0}</em>{2}$</td>
<td>430</td>
</tr>
<tr>
<td>$k_2/(k_{1}^{0} k_{2}^{0})^{1/2}$</td>
<td>0.005</td>
</tr>
<tr>
<td>$i_{lim}^{ox}/\beta \cdot d_0$ (A)</td>
<td>1.5E-7</td>
</tr>
<tr>
<td>$i_{lim}^{red}$ (A)</td>
<td>-1.5±0.3 E-5</td>
</tr>
<tr>
<td>$\beta \cdot d_0$</td>
<td>2</td>
</tr>
</tbody>
</table>

(*) in the EEC(R) model, $E^0_1$ and $E^0_2$ are related to the parameters defined in fig S8 by:

$E^0_1 = E^0_R - RT/F \log(k_1/k_{-1})$, and $E^0_2 = E^0_R - RT/F \log(k'_{1}/k'_{-1})$.

The fits can be parameterized using either $E^0_1$ and $E^0_2$ or $k_1/k_{-1}$ and $k'_{1}/k'_{-1}$. 
References:


