From Enzyme Maturation to Synthetic Chemistry: The Case of Hydrogenases

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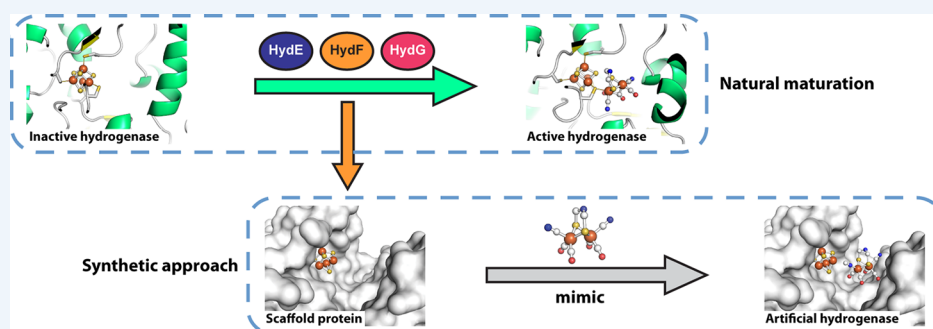
CONSPECTUS: Water splitting into oxygen and hydrogen is one of the most attractive strategies for storing solar energy and electricity. Because the processes at work are multielectronic, there is a crucial need for efficient and stable catalysts, which in addition have to be cheap for future industrial developments (electrolyzers, photoelectrochemicals, and fuel cells). Specifically for the water/hydrogen interconversion, Nature is an exquisite source of inspiration since this chemistry contributes to the bioenergetic metabolism of a number of living organisms via the activity of fascinating metalloenzymes, the hydrogenases.

In this Account, we first briefly describe the structure of the unique dinuclear organometallic active sites of the two classes of hydrogenases as well as the complex protein machineries involved in their biosynthesis, their so-called maturation processes. This knowledge allows for the development of a fruitful bioinspired chemistry approach, which has already led to a number of interesting and original catalysts mimicking the natural active sites. More specifically, we describe our own attempts to prepare artificial hydrogenases. This can be achieved via the standard bioinspired approach using the combination of a synthetic bioinspired catalyst and a polypeptide scaffold. Such hybrid complexes provide the opportunity to optimize the system by manipulating both the catalyst through chemical synthesis and the protein component through mutagenesis.

We also raise the possibility to reach such artificial systems via an original strategy based on mimicking the enzyme maturation pathways. This is illustrated in this Account by two examples developed in our laboratory. First, we show how the preparation of a lysozyme–$\{Mn^I(CO)_3\}$ hybrid and its clean reaction with a nickel complex led us to generate a new class of binuclear Ni-Mn H_2-evolving catalysts mimicking the active site of [NiFe]-hydrogenases. Then we describe how we were able to rationally design and prepare a hybrid system, displaying remarkable structural similarities to an [FeFe]-hydrogenase, and we show here for the first time that it is catalytically active for proton reduction. This system is based on the combination of HydF, a protein involved in the maturation of [FeFe]-hydrogenase (HydA), and a close mimic of the active site of this class of enzymes. Moreover, the synthetic $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ (adt^{2-} = aza-propanedithiol) mimic, alone or within a HydF hybrid system, was shown to be able to maturate and activate a form of HydA itself lacking its diiron active site. We discuss the exciting perspectives this "synthetic maturation" opens regarding the "invention" of novel hydrogenases by the chemists.

■ INTRODUCTION

It is now well agreed that the development of new technologies for energy storage is a prerequisite for the introduction of large amounts of renewable energies, in particular solar and wind energies, into future human societies.[1] Water splitting into

hydrogen and oxygen, in electrolyzers, is one of the most attractive strategies for energy storage because hydrogen can

eventually release its chemical energy back into electricity in fuel cells.[1] However, there are still a number of scientific and technological issues to be addressed before hydrogen becomes an important energy vector of our economy. In particular, production and oxidation of hydrogen requires efficient catalysts to proceed at significant rates. The solution has been found a long time ago with platinum, but unfortunately, this metal is too rare and too expensive for a large scale development of such technologies.[2]

Interestingly, proton reduction and hydrogen oxidation are reactions of bioenergetic metabolism in a number of living organisms.[3] They are catalyzed by metalloenzymes called hydrogenases, which work with remarkably high catalytic rates close to the thermodynamic reaction equilibrium.[3] About 20 years ago, the first crystallographic structures of the two classes of hydrogenases revealed the details of their metal active sites.[4]

[FeFe]-hydrogenases contain a diiron center with carbon monoxide and cyanide as terminal ligands at each Fe atom and with a bidentate bridging aza-propanedithiol (adt^{2-}) ligand connecting the two Fe atoms (Figure 1). The presence of a
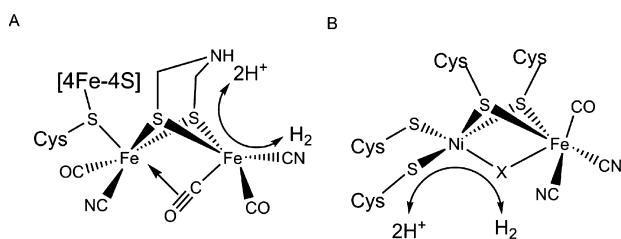
Figure 1. Structure of the active sites of [FeFe]-hydrogenase (A) and [NiFe]-hydrogenase (B).

bridgehead nitrogen atom, essential for proton exchange during catalysis, in this ligand has long been questioned.[5] As related at the end of this Account, we could recently establish it unambiguously. A cysteine, as the unique protein ligand, bridges the diiron center to a [4Fe-4S] cluster. [NiFe]-hydrogenases contain a heterobinuclear Ni−Fe center, in which the Ni and Fe atoms share two cysteine ligands, while Ni completes its coordination sphere with two additional cysteines and Fe with one cyanide and two carbon monoxide ligands (Figure 1). In most hydrogenases, electron transfer between the metal sites and the surface of the enzyme is carried out by chains of iron−sulfur clusters. Maturation of hydrogenases is the process that converts an apohydrogenase into an active holoenzyme through the incorporation of (i) the iron−sulfur centers for electron transport and (ii) the catalytic dinuclear metal centers. Each class of hydrogenase depends on a specific maturation machinery involving a large number of proteins.[3,6] Because hydrogenases rely on non-noble, cheap, and abundant metals, they have been widely studied in the context of potential technological applications, following two major directions.

The first one came from the observation that hydrogenases themselves can be adsorbed on carbon electrodes where they retain their catalytic activities.[7] However, development of such bioelectrodes has been seriously limited for three main reasons. First, hydrogenases are tedious to produce in large quantities. Second, most of them are extremely sensitive to oxygen.[3a] Third, hydrogenases being large molecules (molecular weights above 48 kDa), catalyst densities at electrode surfaces are limited.

The second direction rests on the biomimetic/bioinspired chemistry approach.[8] During the last 20 years, synthetic chemists

have produced a wealth of biomimetic and bioinspired metal complexes.[8b,c] This purely chemical strategy allows the reproduction of structural and functional elements of the natural metal sites within simple and low-molecular weight synthetic organometallic and coordination compounds. For example, dithiolate-bridged diiron compounds[7b,8a,c,9] or heterobimetallic Ni−Fe,[10] Ni−Ru,[11] and Ni−Mn[12] complexes have been reported by us and others[13] to exhibit catalytic activity for proton reduction and, in rare cases, for H_2 oxidation. However, still none of these close mimics could compete against platinum or the hydrogenases themselves. We reasoned that these limitations might originate from the lack of important interactions with polypeptide chains as found in the enzymes. This led us to consider the possibility to improve the performances of the mimics via their incorporation into proteins and thereby to design artificial hydrogenases. The concept of artificial enzymes, combining a polypeptidic chain and a synthetic complex as the active site, finds its origin in the pioneering work of Wilson and Whitesides[14] and has already been applied in many different contexts.[15] Such hybrid systems offer the possibility to rationally tune and optimize the catalytic performances through manipulation of both the synthetic component, by variations of the ligand, and the active site amino acid side chains, through site-directed mutagenesis and more sophisticated directed protein evolution methods. In the case of artificial hydrogenases,[15c,d,16] special attention should be given to optimizing proton and electron transfers, a challenging issue. It is important to note that whereas the choice of the catalytic synthetic component is trivial (since a number of catalytic complexes are available), there is no clear rationale for guiding the choice of the protein, which at minimum should contain a preorganized cavity to selectively bind the catalyst.[16]

The case of [FeFe]-hydrogenases is particularly interesting because their diiron subcluster is attached to the protein by a single cysteine. Thus, we considered, already around 2003, the direct maturation of an apohydrogenase, defined as the enzyme containing all its Fe/S clusters but lacking the diiron subcluster, via simple reaction with appropriate synthetic diiron compounds. This strategy was at that time reminiscent of the well-established semisynthetic maturation of nitrogenases.[17] The project was stimulated slightly later when the physiological [FeFe]-hydrogenase maturation machinery, consisting of the three proteins HydE, HydG, and HydF, was discovered,[18] which was followed by intense research activity aimed at understanding the function of these three proteins.[3a,6]

In this Account, we intend to discuss our various attempts to reach novel hydrogenase-like systems via an original strategy based on mimicking the biosynthetic pathways of the natural metal centers themselves.

FROM MATURATION OF [NiFe]-HYDROGENASES TO A LYSOZYME−{Mn(CO)₃} HYBRID AND Ni−Mn CATALYSTS

Our first goal, targeting artificial hydrogenases, was to prepare biohybrids combining a protein and an organometallic moiety, more specifically one containing CO or CN^- ligand. Only two organometallic biohybrids were structurally characterized then: a {$Re^I(CO)_3(bipy)$}−azurin and a {$Ru^{II}(p$-cymene)}−lysozyme adduct.[19,20] In order to get three-dimensional structures of our synthetic hybrids, we selected lysozyme for its tendency to crystallize. Our initial screening of a variety of iron and manganese carbonyl complexes showed that in most cases binding to lysozyme was not specific enough and no defined

adduct could be isolated. However, the reaction of lysozyme with $[Mn(CO)_3(OH_2)_3]^+$ in acetate buffer, at pH 4.4, yielded a covalent $\{Mn^I(CO)_3\}$–lysozyme adduct, which could be purified.[21] The presence of CO ligands was shown by characteristic infrared ν_{CO} absorption bands. A 1.7 Å resolution three-dimensional crystal structure was obtained (Figure 2)
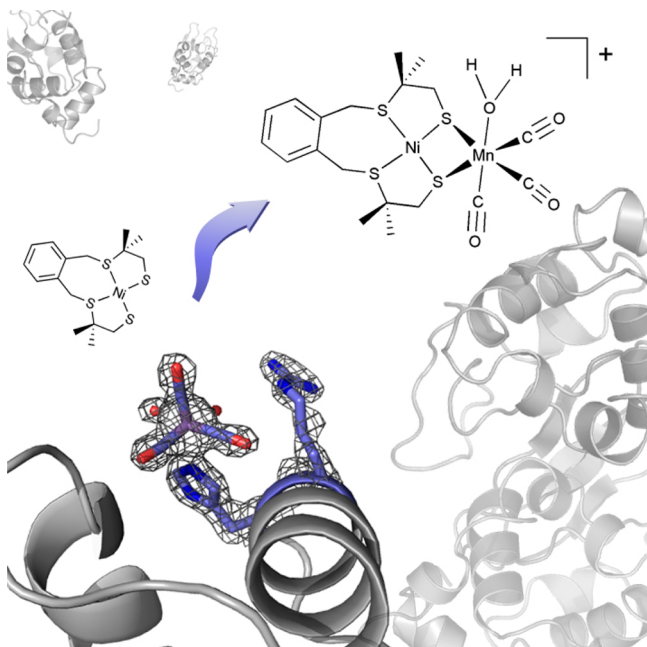
Figure 2. Structure of the manganese–carbonyl site in lysozyme and its reaction with a Ni complex generating a Ni–Mn hydrogenase mimic. Electronic densities show the $\{Mn^I(CO)_3(H_2O)_2\}$ unit bound to a histidine residue of lysozyme (PDB 2Q0M).

revealing binding of one organometallic moiety, $\{Mn(CO)_3(OH_2)_2\}^+$, to the Nε of the His15 side chain of the protein (Mn–N distance = 2.4 Å), as in the arene–ruthenium derivative previously reported.[20] The manganese ion has octahedral coordination with three CO ligands in facial arrangement and two aquo ligands *cis* to each other.

A parallel could be made with the natural [NiFe]-hydrogenase maturation process.[3b] A $\{Fe(CO)(CN)_2\}$ organometallic unit is proposed to be assembled within the HypD/HypC complex and transferred to the large subunit of the hydrogenase. Then, a nickel ion is transferred from the Ni-binding proteins, HypA and HypB, thanks to the GTPase activity of HypB, to generate the Ni–Fe active center (Figure 3). Inspired by this simple synthetic process, we tried to exploit the $\{Mn^I(CO)_3\}$–lysozyme adduct for preparing an artificial hydrogenase combining lysozyme and a

Ni–Mn center. Reaction with a Ni complex such as [Ni(xbsms)] (H$_2$xbsms = 1,2-bis(4-mercapto-3,3-dimethyl-2-thiabutyl)-benzene) did not yield the expected hybrid. Instead the reaction yielded free lysozyme and a novel complex, [Ni(xbsms)Mn-$(CO)_3(OH_2)]^+$ with good yields (Figure 2).[21] Interestingly this reaction applies to other Ni complexes since incubation of the hybrid Mn(CO)$_3$–protein with smaller nickel thiolate complexes such as $[Ni(emi)]^{2-}$ and $[Ni(ema)]^{2-}$ (H$_2$emi = N,N'-ethylenebis(2-mercaptoisobuyramide), H$_2$ema = N,N'-ethylenebis(2-mercaptoacetamide)) also cleanly yielded binuclear Ni–Mn complexes. By comparison, reaction of [Mn-$(CO)_3(OH_2)_3]^+$ with [Ni(xbsms)] under the same conditions is far less selective, yielding a mixture of products. Even though it failed to produce an artificial hydrogenase, this study provided several interesting outcomes. The Mn–lysosyme adduct was the first structurally characterized tricarbonylmanganese(I)–aquo complex with a monodentate organic ligand. Additionally, it showed that coupling a Mn carbonyl compound to a Ni coordination complex is better controlled within protein scaffolds. Finally, the $[Ni(xbsms)Mn(CO)_3(OH_2)]^+$ complex was the first Ni–Mn complex mimicking the active site of NiFe-hydrogenases, opening the way toward a novel class of bioinspired catalysts for H$_2$ production.[12,13e] [Ni(xbsms)Mn-$(CO)_3(OH_2)]^+$ is active for H$_2$ evolution from trifluoroacetic acid in DMF with an overpotential requirement of 860 mV, a value comparable to that for a related Ni–Fe mimic[10] but higher than those displayed by Ni–Ru systems.[11d] A combined electrochemical and theoretical (DFT) study indicated a heterolytic mechanism for hydrogen evolution from a hydride derivative (Figure 4).[12] The structure of the active intermediate, with a bridging hydride ligand between Ni and Mn, resembles that of the Ni–C active state of NiFe hydrogenases.[3a] More recently, Song and co-workers reported another series of active Ni–Mn mimics of the active site of [NiFe]-hydrogenases based on a $\{Mn^I(CO)_3\}$ moiety.[13e]

■ FROM MATURATION OF [FeFe]-HYDROGENASES TO ARTIFICIAL HYDROGENASES

Biosynthesis of the active site of [FeFe]-hydrogenases, HydA, has recently been the subject of intense research and is now at least partly clarified.[3,6,22] Briefly, the electron-channeling Fe/S-clusters are first assembled within the protein (Figure 5, step 1). Then the dinuclear $\{Fe_2(adt)(CO)_3(CN)_2\}$ complex is synthesized and inserted into HydA via the concerted effort of at least three maturase proteins, all of which are Fe/S proteins (Figure 5, step 2). HydG is a radical-SAM (S-adenosylmethionine) enzyme that is responsible for the synthesis of carbon monoxide and cyanide molecules via decomposition of a tyrosine substrate. Recent structural and spectroscopic data suggest that
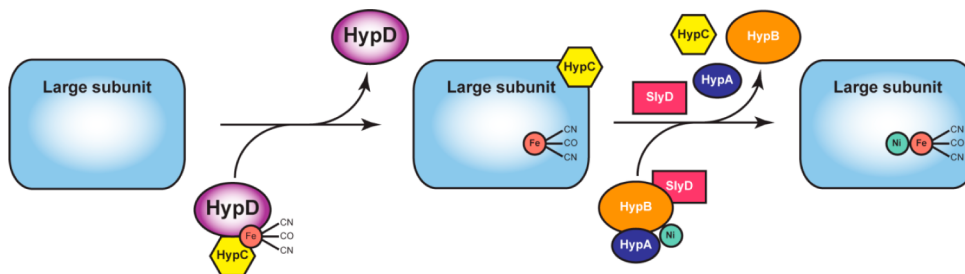
Figure 3. [NiFe]-hydrogenase maturation. An organometallic Fe complex is assembled in HypD/HypC, transferred to the large subunit of the hydrogenase and combined to a Ni ion provided by the HypA-HypB-SlyD complex.
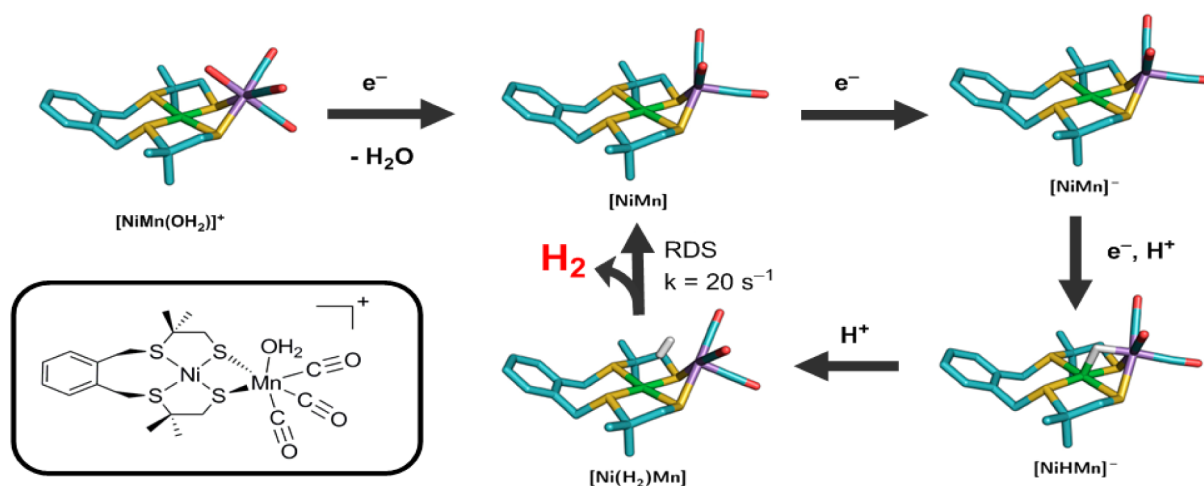
Figure 4. H$_2$ evolution mechanism catalyzed by a synthetic Ni–Mn mimic (RDS = rate-determining step).
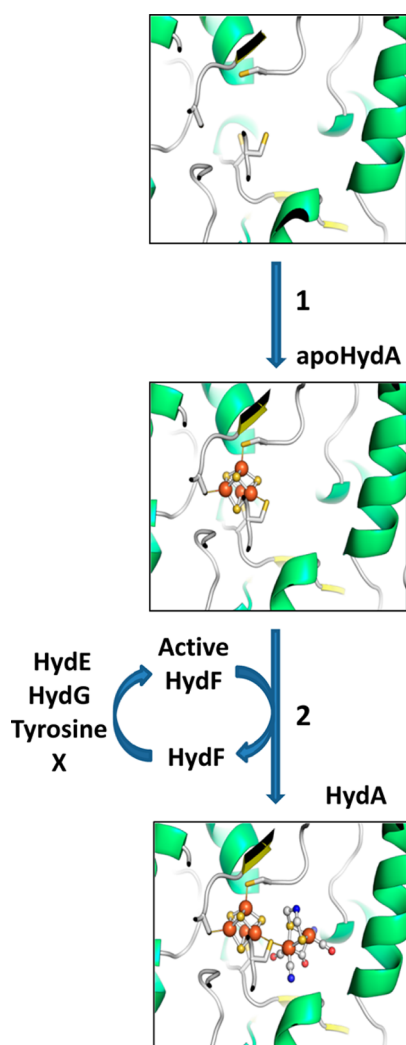

Figure 5. [FeFe]-hydrogenase maturation. Step 1, assembly of Fe–S clusters into HydA (adapted from PDB 3C8Y). Step 2, insertion of the diiron subunit from HydF containing the diiron precursor, which derives from HydE/HydG activity. Tyrosine is the substrate of HydG. X is the unknown substrate of HydE.

these diatomic molecules are maintained transiently attached to HydG in the form of a still ill-defined protein-bound {Fe(CO)$_x$(CN)$_y$} center, coupled to a [4Fe-4S] cluster of HydG and waiting for transfer to the HydF protein. Then HydE, also a radical-SAM enzyme, is supposed to be responsible for the synthesis of the aza-propanedithiol (adt^{2-}) ligand from a still unknown substrate and its transfer to HydF. HydF is thus proposed to function as a scaffold protein assembling the bricks provided by HydG and HydE to generate a precursor of the diiron subunit of an active [FeFe]-hydrogenase. Indeed, the final step of the maturation process consists in the simple transfer of the diiron precursor from HydF (active HydF in Figure 5) to HydA where it acquires the chemical and structural organization required for full activity. The structure of that final diiron precursor in active HydF is not known, but it is well established that it contains all the elements, in the appropriate configuration, to allow full maturation of HydA without any extra reactant.

On the basis that HydF is set for binding a {Fe$_2$(adt)-(CO)$_x$(CN)$_y$} subunit and with the objective of constructing an artificial [FeFe]hydrogenase, we reasoned that HydF would be the perfect protein host for a synthetic bioinspired organometallic diiron complex. This led us first to extensively characterize pure preparations of HydF from *Thermotoga maritima*. In particular we established that its unique [4Fe-4S] cluster was coordinated by only three conserved cysteines, leaving one Fe atom accessible to exogenous ligands such as imidazole or a histidine of an N-terminal histidine tag, as shown by HYSCORE experiments.[23] Then we showed that indeed stable hybrid 1:1 complex–protein adducts could be obtained by reacting HydF with a small excess of the biomimetic compound {Fe$_2$(L)(CO)$_4$(CN)$_2$} (L = adt^{2-}, propanedithiol (pdt^{2-}), or oxa-propanedithiol (odt^{2-})).[24] Furthermore, using this methodology, the hybrid could be labeled with ^{13}CO and C^{15}N$^-$ ligands. The structure of the metallocluster of the HydF hybrid has been unambiguously established by a variety of spectroscopic and computational methods (Figure 6). First, the presence of CN$^-$ and CO molecules in the hybrid could be demonstrated, when W. Lubitz and E. Reijerse in Mülheim (Germany), who have an outstanding expertise in spectroscopic characterization of hydrogenases, were invited to characterize the protein by FTIR spectroscopy. Furthermore, S. Gambarelli and J.-M. Mouesca, in Grenoble, were able to show by X-band EPR spectroscopy that the intact diiron complex was interacting with the [4Fe-4S] cluster. More specifically, X-band two-dimensional pulsed electron spin echo envelope modulation (ESEEM) spectroscopy using HydF hybrid preparations labeled with ^{13}CN$^-$ ligands
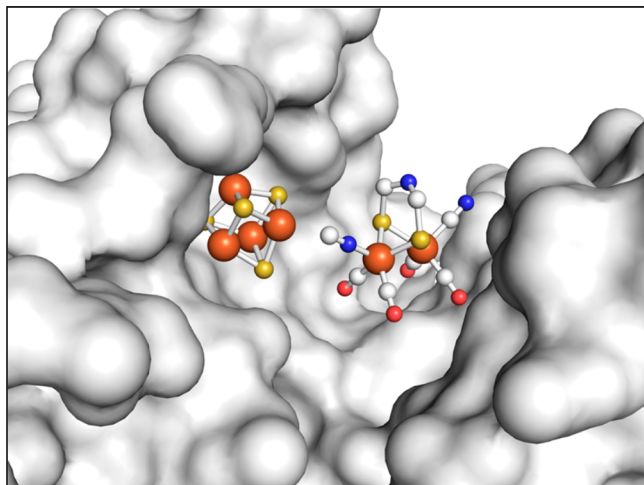
Figure 6. Structural model of the HydF hybrid based on the apoHydF structure from *T. neopolitana* (PDB 3QQ5). The 6Fe center, whose structure has been determined spectroscopically,[24] was docked into the putative active site.

demonstrated that the diiron complex was binding to the protein through only one coordination bond: a cyanide bridge connecting one Fe of the complex and one Fe of the [4Fe-4S] cluster, consistent with the previously established ability of the cluster to bind exogenous ligands. Furthermore, computation of hyperfine coupling constants indicated that the cyanide C atom was bound directly to the cluster while its N atom was bound to the diiron complex, implying precedented cyanide linkage isomerism. The resulting hexairon cluster, shown in Figure 6, is highly reminiscent of the HydA active site, even though there are significant differences. The HydA dinuclear Fe unit indeed contains only three CO ligands and one is bridging. Furthermore, it is connected to a [4Fe-4S] cluster via a bridging cysteine ligand. These results are remarkable because they show how chemists can generate hybrid proteins with great structural similarity with the target metalloenzyme, and they provide the first example of a rational structural design of an artificial hydrogenase. They also illustrate the potential of having a synthetic component in the hybrid as it was readily possible to produce hybrids with a variety of dinuclear iron complexes, containing for example labeled ligands or analogues of adt^{2-} (pdt^{2-} and odt^{2-}).

Interestingly, the $[Fe_2(pdt)(CO)_4(CN)_2]^{2-}$/HydF hybrid displayed a small but significant hydrogenase activity (Figure 7), while the adt analog proved inactive. This was shown in a standard chemical assay (Figure 7A), using sodium dithionite and methyl viologen as the source of electrons, as well as in a photochemical assay (Figure 7B), using a metal–organic photosensitizer and a sacrificial electron donor under visible light irradiation (>400 nm). The reaction starts immediately and proceeds with a significant initial rate (TOF $\approx 0.5-1$ min^{-1}) but stops after a few minutes. Experimental details are given in the Supporting Information. The TOF value for a HydA preparation reconstituted with $[Fe_2(pdt)(CO)_4(CN)_2]^{2-}$ was only about 50-fold larger.[25] Even though these activities are very low, the $[Fe_2(L)(CO)_4(CN)_2]^{2-}$/HydF synthetic metalloprotein provides an opportunity to progress toward more efficient artificial hydrogenases, notably through rational site directed mutagenesis or directed evolution methodologies in combination with synthetic manipulation of the diiron subunit. Moreover, it will allow us to unravel the effects of protein scaffolds on the activity of small organometallic complexes. Obviously, a three-dimensional structure of the HydF hybrid is highly requested for the success of such a project.

■ SEMISYNTHETIC MATURATION OF [FeFe]-HYDROGENASES

There was another unexpected outcome of this synthetic work. In line with HydF's native function as the mediator of HydA maturation, we checked whether it could transfer the hybrid-bound synthetic components to the apoprotein form of HydA (apoHydA), whose active site contains only the [4Fe-4S] cluster and lacks the diiron subunit.[24] Thomas Happe at Böchum University (Germany) was invited to join our efforts, during the 2012 Solar Fuel Gordon Research Conference in Italy. T. Happe's group has a unique expertise in purification of hydrogenases and in assaying hydrogenase activities. In preliminary experiments in Grenoble, we were able to show strong activation of apoHydA from *Chlamydomonas reinhardtii* provided by T. Happe. Then combining FTIR characterization in Muelheim and activity assays in Böchum, we could firmly establish that indeed the diiron complex of the HydF hybrid could be transferred into the inactive apoHydA (Figure 8). Remarkably, the resulting HydA hybrid displayed an activity comparable to that of the natural enzyme, however, exclusively when treated
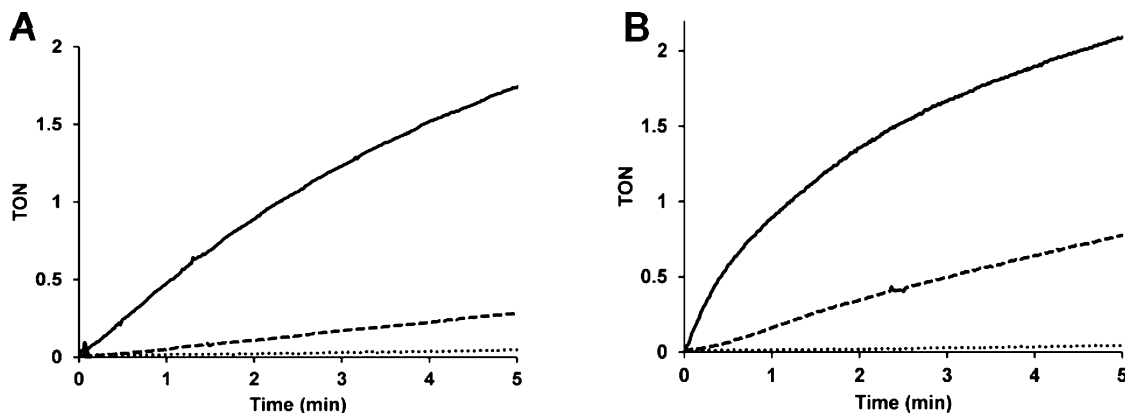
Figure 7. Hydrogen evolution catalyzed by the HydF hybrid. (A) Chemical assay using sodium dithionite/methyl viologen as the reducing agent. (B) Photochemical assay using $[Ru(bpy)_3]^{2+}$ as photosensitizer and sodium ascorbate as sacrificial electron donor. Experimental conditions are described in Supporting Information. HydF hybrid (full), free $[Fe_2(pdt)(CO)_4(CN)_2]^{2-}$ complex (dashed), and apoHydF (dotted). TON is defined as mol(H$_2$)·mol(cat)$^{-1}$.
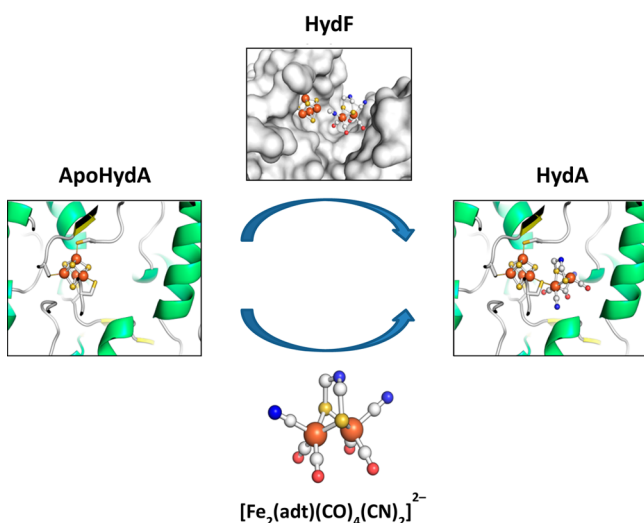
Figure 8. Synthetic maturation of [FeFe]-hydrogenase by the HydF hybrid (upper arrow) and by the $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ complex (lower arrow).

with the HydF hybrid containing the adt^{2-} ligand, even though other complexes (with pdt^{2-} and odt^{2-} ligands) were also transferred to HydA. Subsequent experiments showed that full activation also occurred in the absence of HydF, simply through treatment of apoHydA exclusively with synthetic $[Fe_2(adt)-(CO)_4(CN)_2]^{2-}$ (Figure 8).[26] In that case, it was shown that the methodology could be applied to various [FeFe]-hydrogenases, as shown from the observed full activation of HydA not only from *C. reinhardtii* but also from *Megasphera elsdenii* and from *Clostridium pasteurianum*, providing experimental substance to the concept of synthetic maturation of hydrogenases designed almost 10 years before.

These results have a number of fascinating implications. First, they provide a final and unambiguous demonstration for the presence of a nitrogen atom within the bridging dithiolate ligand, an issue previously left with some ambiguity.[5] Second, they show that the reaction between the HydF hybrid and apoHydA generates an active diiron site within HydA identical to the naturally biosynthesized one. Thus, it appears that, during the transfer process, the $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ subunit of the HydF hybrid spontaneously enjoys the necessary transformations requested to convert it into the natural subunit: (i) substitution of a CO ligand by a cysteine ligand of the apoHydA [4Fe-4S] cluster, as confirmed by the release of free CO during the reaction; (ii) formation of a CO-bridged rotated state; (iii) generation of an open coordination site for proton or H_2 activation. Third, these results show how a protein environment can convert a catalytically quite inactive molecular complex into a remarkably active catalyst. Even though there is a bias in the demonstration since the chosen host protein is an apohydrogenase, which has been designed over billions of years of evolution to selectively optimize the hydrogenase activity of such a diiron subunit, it provides an exquisite validation of the general "artificial enzyme" strategy. Fourth, the HydF hybrid is so efficient in activating apoHydA that it is tempting to suggest that the structure of the still unknown diiron precursor naturally assembled on HydF (active HydF in Figure 5) is identical to the $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ complex linked to the [4Fe-4S] cluster via a CN^- ligand found in the hybrid. Now on the basis of the readily accessible spectroscopic signatures, in particular with FTIR spectroscopy, the preparation of hybrid HydF

constructs should greatly facilitate the characterization of the native Fe_2–HydF precursor. Experiments along this direction are in progress in our laboratory.

Finally this remarkably simple "chemical" HydA activation process opens a wide variety of potential applications that are briefly developed in the following. First, it provides a unique experimental tool allowing high throughput screening for efficient and oxygen-resistant hydrogenases in microorganisms, which would be more suitable for technological applications. To date only a subset of HydA enzymes have been analyzed owing to the difficulty of generating sufficiently large amounts of fully active enzymes. Now, it becomes possible to analyze a broad spectrum of HydA sequences using standard protein expressing strains such as *Escherichia coli*, even though they lack the HydA maturation machinery. This allows an easy production and analysis of stable apoHydA preparations since the latter can be readily activated by a single reaction with the synthetic $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ complex. Second, fine modifications can be easily brought to the diiron site via chemical synthesis of the appropriate complex and incorporation into apoHydA. For example, introduction of the $[Fe_2(pdt)(CO)_4(CN)_2]^{2-}$ and $[Fe_2(odt)(CO)_4(CN)_2]^{2-}$ complexes into HydA, as well as isotope-labeled versions such as $[Fe_2(pdt)(CO)_4(C^{15}N)_2]^{2-}$ and $[Fe_2(pdt)(CO)_4(^{13}CN)_2]^{2-}$ complexes was possible. We will briefly discuss these two examples in the following.

First, the characterization of the $[Fe_2(pdt)(CO)_3(CN)_2]/$ HydA (pdt-HydA) assembly nicely shows how the nitrogen atom of the dithiolate bridge is crucial not only for activity, since pdt-HydA lacking this essential proton exchange site is essentially not enzymatically active, but also for the redox properties of the cluster and its reactivity toward CO.[27] Indeed, in contrast to the natural site, which displays three redox states during titration, pdt-HydA can access only two that can be characterized by FTIR spectroscopy. Furthermore, the latter is much more resistant at high redox potential and to inhibition by CO.[27] Second, taking advantage of the possibility to obtain a pure redox state in the case of pdt-HydA, which allows easy spectroscopic characterization, in contrast to the adt-HydA, which is usually obtained as a mixture of redox forms, we produced $C^{15}N^-$ and $^{13}CN^-$ labeled pdt-HydA. W. Lubitz and collaborators performed HYSCORE and ENDOR on the $S = 1/2$ oxidized form, which displays an FTIR spectrum resembling the active H_{ox} state of the native protein. ^{13}C and ^{15}N hyperfine couplings could be determined with high resolution and assigned, showing delocalization of the spin density over the two Fe atoms of the diiron center.[28] Access to the relative orientations of the nitrogen and carbon hyperfine tensors led to the definition of a **g**-tensor orientation following the local symmetry of the binuclear center in which the Fe–Fe bond represents one of the **g**-principle axes. Comparable data were obtained using HydA from *Clostridium pasteurianum* labeled with $C^{15}N^-$ and $^{13}CN^-$ via *in vitro* maturation by HydE, HydF, and HydG in combination with ^{13}C and ^{15}N labeled tyrosine, the precursor of CO and CN^- ligands.[29]

Obviously, more extensive synthetic modifications are possible, through incorporation of a variety of mimics into apoHydA, which might generate new hydrogenase active sites, not selected by Nature before. An initial attempt has been recently made by W. Lubitz's group using a variety of modifications within the mimic.[25] Even though no synthetic analog could compete against the natural composition, this study showed that it is possible, for example, to vary the proportions of CO and CN^- ligands or to methylate the dithiolate ligand while

retaining significant activity. Continued work along these lines with the "invention" of new hydrogenases by "synthetic maturation" of HydA is a highly exciting perspective.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods for HydF expression and purification, preparation of the hybrid protein, and hydrogenase activity assay. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.accounts.5b00157.

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The authors declare no competing financial interest.

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