Hydrogenases: Hydrogen-Activating Enzymes

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biohydrogen · bioorganometallic chemistry · hydrogenases · hydrogen-producing catalysts · metalloenzymes

1. Introduction

Many microorganisms, such as methanogenic, acetogenic, nitrogen-fixing, photosynthetic, or sulfate-reducing bacteria, metabolize hydrogen.[1, 2a] Hydrogen activation is mediated by a family of enzymes, termed hydrogenases,[3] which either provide these organisms with reducing power from hydrogen oxidation or act as "electron sinks", following the reaction: $H_2 \rightleftharpoons 2H^+ + 2e^-$. Not surprisingly, hydrogenases are mostly studied with a view to designing chemical or biochemical processes to produce molecular hydrogen more abundantly and cheaply than with platinum catalysts; molecular hydrogen is an ideally clean fuel.[4, 5]

Hydrogenases (cytochrome c_3 oxidoreductase, EC 1.18.99.1) are classified into two major families in the present paper on the basis of the metal content of their respective dinuclear catalytic centers, that is nickel – iron (NiFe) hydrogenases[6] and "iron only" (FeFe) hydrogenases.[2] Some NiFe hydrogenases also contain selenium at their catalytic center in the form of selenocysteine (Table 1).[6a] The two hydrogenases families differ functionally from each other in that NiFe hydrogenases tend to be more involved in hydrogen oxidation and FeFe hydrogenases in hydrogen production. Moreover, NiFe hydrogenases are approximately $10^{-1} - 10^{-2}$ times less active, show 10^2 times more affinity for hydrogen, and are less sensitive to inhibition by oxygen and carbon monoxide than FeFe hydrogenases (Table 2).[2a] A "metal-free" hydrogenase, found in methanogenic bacteria, catalyzes the reversible reduction of a methenyltetrahydromethanopterin (methenyl-H_4MPT) methanogenic cofactor with H_2 to form methylene-H_4MPT and a proton during methane formation from CO_2 and $4H_2$.[7]

Table 1. *Physico-chemical properties of typical hydrogenases.*[a]

Family: Genus and species:	NiFe *D. gigas*	NiFeSe *Dm. baculatum*	FeFe *D. desulfuricans*
cell localization	periplasm	periplasm	periplasm
molecular mass [kDa]	89.5	85	53
no. of subunits	2	2	2
no. of iron atoms	12	14	14
no. of nickel atoms	1	1	
other atoms	1 magnesium	1 selenium	
nonproteic ligands	2 CN, 1 CO	2 CN, 1 CO	2 CN, 3 CO, 1 di(thiomethyl)amine[b]
no. of $[4Fe-4S]^{2+/1+}$ clusters	2	3	3
no. of $[3Fe-4S]^{2+/1+}$ clusters	1		

[a] *D.* = *Desulfovibrio*, *Dm.* = *Desulfomicrobium*. [b] The assignment of the di(thiomethyl)amine is tentative.[17]

Table 2. *Hydrogenases' catalytic activities.*

	NiFe hydrogenases	FeFe hydrogenases
H_2 production[a]	700	6000 – 9000
H_2 consumption[a]	700	28000

[a] Measured as molecules of hydrogen per second per hydrogenase molecule at 30 °C.

The three-dimensional atomic models of four NiFe,[8–11] one NiFeSe,[12] and, more recently, two FeFe[13, 14] hydrogenases (Table 3) have been elucidated by X-ray crystallography on the basis of gene sequencing[15] and a wealth of biochemical and spectroscopic studies, in some cases coupled with isotopic labeling.[6, 2, 16] These results represent a considerable impetus to research the catalytic mechanism of hydrogenases and the design of organometallic compounds which mimic their structural or functional properties, or both. The aim of this short review is to highlight some recent works and trends in hydrogenase research.

2. Atomic Architectures

The atomic architectures of NiFe hydrogenases, including the NiFeSe ones, show great similarities, in accordance with several conserved motifs in the amino acid sequences.[6a] The archetypal *Desulfovibrio gigas* NiFe hydrogenase (Figure 1a),[8] is a globular heterodimer (with a radius of about 3 nm) consisting of a small (relative molecular mass (M_r): 26 kDa) and a large subunit (M_r: 63 kDa); these interact extensively with each other (around 3500 Å^2). The large subunit contains a moiety, deeply buried inside the protein, including an Ni–Fe dinuclear center and three nonproteic ligands, one CO and two CN^- ligands, attached to the iron atom (Figure 1b). The small subunit contains three iron –

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Table 3. *NiFe and FeFe hydrogenases: X-ray structures.*

Organism and functional state of the enzyme[a]	Class of enzyme	Ref.	Resolution [Å]	PDB file[b]
D. gigas	NiFe			
unready		[8a]	2.85	1FRV
unready		[8b]	2.54	2FRV
active		[8c]	2.7	t.b.s.[c]
D. vulgaris[d]	NiFe			
ready		[9a]	1.8	1H2A
active		[9b]	1.4	1H2R
D. fructosovorans	NiFe			
unready		[10a]	2.7	1FRF
		[10b]	1.8	t.b.s.[c]
Dm. baculatum	NiFeSe			
active		[12]	2.15	1CC1
D. desulfuricans	NiFe			
unready ?[e]		[11]	1.8	1E3D
C. pasteurianum	FeFe			
oxidized ?[e]		[13]	1.8	1FEH
CO-inhibited		[18]	2.4	1C4A
D. Desulfuricans	FeFe			
mixed		[14]	1.6	1HFE
reduced		[17]	1.85	t.b.s.[c]

[a] *D.* = *Desulfovibrio*; *Dm.* = *Desulfomicrobium*; *C.* = *Clostridium*; unready = inactive oxidized forms; activated after a prolonged exposure to H_2; ready = inactive oxidized forms, immediately active after exposure to H_2; active = reduced forms. [b] Protein Databank file deposition code, see: http://www.rcsb.org. [c] t.b.s. = to be submitted. [d] The structure of a CO complex has been solved at 1.4 Å but has not yet been published.[9c] [e] ? = the redox state of the enzyme is not reported.

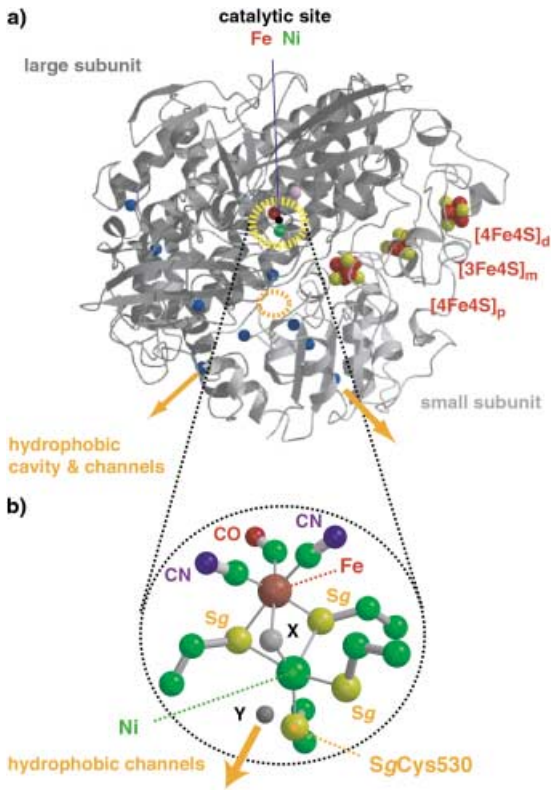

Figure 1. *NiFe hydrogenases. a) Overall structure. Structure of the* D. gigas *enzyme.[8] The polypeptide folds of the large and small subunits are colored in dark and light grey respectively. The catalytic center Ni–Fe (dotted yellow circle and (b)) and iron–sulfur cluster atoms are represented by spheres. A hydrophobic cavity (dotted orange circle) and channels (not shown) have been probed by diffusing xenon atoms within protein crystals.[10] The cavity and channels might mediate molecular hydrogen transfer between the surface and the catalytic center.[10] The orange arrows identify probable exits (or entrances) for H_2. Color code for (a): iron = red spheres, nickel = green, sulfur = yellow, xenon = blue, magnesium = purple. b) Close-up view of the catalytic site. The Ni–Fe bridging atom (X) is thought to be an oxo, hydroxo[8, 10] or sulfur[9a, 11] species in the oxidized inactive forms of the hydrogenase. All these species are absent in the reduced potentially active forms.[9b, 12] The vacant axial nickel coordination site (Y) is close to one end of a hydrophobic channel. Only one of the four cysteic ligands, Cys 530 (SeCys in* Dm. Baculatum*),[12] has been labeled. Color code for (b): iron = large red sphere, nickel = large green, sulfur = yellow, carbon = small green, nitrogen = blue, oxygen = small red. The figure was prepared with the Molscript[47a] and Raster3D[47b] programs from the Protein Databank file 2FRV.*

sulfur clusters: one proximal (p) [4Fe–4S] at 1.4 nm from the dinuclear center, one [3Fe–4S] termed as medial (m), and a distal (d) [4Fe–4S] cluster close to the molecular surface. The clusters centers are 1.2 nm apart from each other. The molecule also contains a large cavity and channels, lined with hydrophobic amino acid side-chains, that interconnect the dinuclear Ni–Fe center and the molecular surface.[10] Many hydrogen-bonded water molecules sites are present on the interior and "cover" the surface of the protein.

The two known FeFe hydrogenase atomic structures, from the bacteria *Clostridium pasteurianum* and *D. desulfuricans*, respectively, show a common core, which contains a moiety, deeply buried inside the protein, with an Fe–Fe dinuclear center, nonproteic bridging, terminal CO and CN^- ligands attached to each of the iron atoms, and a dithio moiety, which also bridges the two iron atoms and has been tentatively assigned as a di(thiomethyl)amine (Figure 2).[17] This common core also harbors three [4Fe–4S] iron–sulfur clusters.[13, 14, 17] The *C. pasteurianum* hydrogenase contains two additional domains, each with one iron–sulfur cluster, which gives the molecule a mushroom aspect.[13] In FeFe hydrogenases, as in NiFe hydrogenases, the set of iron–sulfur clusters is dispersed regularly between the dinuclear Fe–Fe center and the molecular surface. These clusters are distant by about 1.2 nm from each other but the [4Fe–4S] cluster closest to the dinuclear center is covalently bound to one of the iron atoms (usually termed Fe1) though a thiolate bridging ligand (Figure 2). The moiety including the dinuclear center, the thiolate bridging ligand, and the proximal [4Fe–4S] cluster is known as the "H-cluster".[2a] Here again, a channel, lined with hydrophobic amino acid side chains, nearly connects the dinuclear center and the molecular surface.[14] Furthermore hydrogen-bonded water molecule sites have been identified at the interior and at the surface of the protein.

The catalytic sites

It is now clearly confirmed that the catalytic sites of both NiFe and FeFe hydrogenases include an organometallic moiety consisting of their respective dinuclear Ni–Fe or Fe–Fe centers and nonproteic CN^- and CO ligands attached to the iron atoms (Figure 1 b, Figure 2);[8, 12–14] for FeFe hydrogenases, another nonproteic moiety, bridging the two iron atoms has been

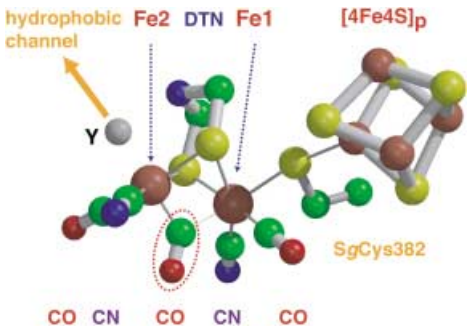

Figure 2. *FeFe hydrogenase catalytic site.[13, 14] The CO circled by a dotted line bridges Fe1–Fe2 in the oxidized form and binds terminally to Fe2 in the reduced one.[17] The small organic moiety bridging Fe1–Fe2 has been tentatively assigned to be a di(thiomethyl)amine (DTN).[17] The vacant axial coordination site (Y) of the Fe2 atom is close to one end of a hydrophobic channel[14, 22] and binds CO, an inhibitor of hydrogenase.[18] Color code: iron = large red spheres, sulfur = yellow, carbon = green, nitrogen = blue, oxygen = small red. The figure was prepared with the Molscript[47a] and Raster3D[47b] programs from the Protein Databank files 1FEH and 1HFE.*

tentatively identified as a di(thiomethyl)amine.[17] It should be emphasized that the presence of the Fe(CO)(CN)$_x$ moiety(ies) (where $x = 1, 2$) in both the NiFe and FeFe hydrogenases and the bidentate bridge in FeFe hydrogenases is unprecedented in biology. In NiFe hydrogenases, the dinuclear Ni–Fe center is bound to the protein through four cysteic sulfur ligands and four hydrogen bonds mediated by the two cyanide ligands (Figure 1 b). By contrast, the dinuclear Fe–Fe center of FeFe hydrogenases is only bound to the protein through the thiolate ligand between one of the iron atoms (Fe1) and the closest [4Fe–4S] cluster and three hydrogen bonds involving the cyanide ligands (Figure 2).

The oxidized and reduced structures of NiFe hydrogenase catalytic site differ in that a μ-(hydro)oxo[8] or SO[9a] bridging Ni and Fe in the oxidized form is no longer present in the reduced one (Figure 1 b).[12, 9b] In addition, Ni and Fe are closer to each other (0.25 nm versus 0.29 nm) in the reduced forms so that metal hydride bridging is made possible (see below). In the oxidized enzyme,[8] the coordination of the nickel is distorted square pyramidal and the iron shows an octahedral conformation. One bridging cysteic sulfur atom (Figure 1 b) is an apical ligand to the nickel atom, whereas the three other cysteic sulfur ligands and the small exogenous μ-(hydro)oxo bridging ligand are bound approximately in plane. The catalytic site architectures of the *Desulfomicrobium* (*Dm.*) *baculatum* NiFeSe hydrogenase and *D. gigas* NiFe hydrogenase are similar to each other but for the substitution of one terminal sulfur ligand (*Sg*Cys 530) in *D. gigas* by a cysteic selenium ligand (SeCys 487) in *Dm. baculatum*. It is of interest to note that the *D. gigas* *Sg*Cys 530 and its equivalent are slightly "disordered" in the crystal structures (see below).

Joint crystallographic and infrared spectroscopy analyses of an oxidized and a reduced form of the *D. desulfuricans* FeFe hydrogenase have revealed that, in the oxidized form, one CO bridges the two iron atoms at the catalytic site (termed Fe1 and Fe2, respectively; Figure 2) whereas in the reduced form this same CO is terminally bound to the iron atom Fe2.[14, 17] In both the *C. pasteurianum* and *D. desulfuricans* oxidized FeFe hydrogenases, Fe1 has six ligands in a distorted octahedral conformation and Fe2 has five ligands and a sixth open site, which is apparently empty in the partially reduced or reduced *D. desulfuricans* hydrogenase and occupied by a putative water molecule[13] or a CO ligand[18] in the oxidized or CO-inhibited *C. pasteurianum* enzyme (Y in Figure 2). It is also of interest to note that all the CO and CN ligands are on the other side of the Fe–Fe center with respect to the Fe–Fe bridging dithio moiety (DTN in Figure 2).

Access to the catalytic sites

As above underlined, the catalytic sites of both NiFe and FeFe hydrogenases are deeply buried inside the respective proteins. It follows that the components of the catalytic reaction (that is, electrons, hydrons, and molecular hydrogen) have to shuttle, over several nanometers, between these sites and the molecular surface.

Electrons most probably use iron – sulfur clusters as relays, as evidenced by many redox titrations and spectroscopic studies of NiFe[6a] and FeFe[2a] hydrogenases upon hydrogen activation. This goes along with the redox center's arrangement and proximity (< 1.4 nm), observed in the crystal structures, which should favor fast electron transfer whatever the postulated mechanism is through-space[19a] or through bonded orbitals with occasional through-space jumps.[19b]

Hydrons are known to move in proteins through small (about 0.1 nm) and fast (10^{-9} s) displacements mediated by rotational movements of donor and acceptor groups (including histidines, carboxylate groups, acidic residues with suitable pK_a values, or water molecules).[20] Several experimental evidences, such as the above-mentioned crystallographic disorder of the cysteic sulfur[8] or selenium ligand[12] on the nickel, suggest that, at the outset (or entrance) of the NiFe and FeFe hydrogenase catalytic sites, hydron pathways most probably involve a basic side chain (see below). This could be: a) one nickel terminal cysteic sulfur ligand, *Sg*Cys 530 in the *D. gigas* NiFe enzyme[21] or SeCys 487 in the *Dm. baculatum* NiFe enzyme [12] or b) a lysine residue close to Fe2 in the FeFe enzymes.[22] Beyond (or before) that "point", several plausible proton pathways between the catalytic site and the molecular surface of the *D. gigas* NiFe[21] and the two FeFe hydrogenases[22] have been proposed. However these hypotheses have yet to be confirmed by additional experimental evidence, which could be provided, for example, by genetic engineering (site-directed mutagenesis).

Molecular hydrogen, a small diffusible molecule, was first thought to access or exit from the catalytic site of NiFe hydrogenase through multiple dynamic pathways depending on fluctuations in the protein conformation.[8a, 23] However, further crystallographic analysis and molecular dynamics calculations have strongly suggested that molecular hydrogen could in fact use the hydrophobic cavity and channels network, observed in these NiFe hydrogenases, to shuttle between the catalytic site and the molecular surface.[10] By analogy, it has been proposed that, in FeFe hydrogenases, molecular hydrogen also shuttles through the unique hydrophobic channel interconnect-

ing the active site and the molecular surface.[14, 22] These hypotheses seem all the more plausible as in NiFe and FeFe hydrogenases one end of these hydrophobic channels is close to the putative H_2 binding site at the respective catalytic centers, that is, the nickel and Fe2 atoms (Y in Figure 1b and 2).

3. Enzymatic Activities and Redox Chemistry

The catalytic activities of NiFe hydrogenases are usually measured by spectroscopy, gas chromatography, and electrochemistry with various electron acceptors or donors (Table 2).[24] Moreover, hydrogenases (E) can convert *para* hydrogen (antiparallel nuclear spins) into *ortho* hydrogen (parallel nuclear spins) following the global reaction: $E + H_2(\uparrow\downarrow) + H_{b+} \rightarrow E + H_2(\uparrow\uparrow) + H_a^+$, where H_a^+ and H_b^+ are provided by H_2 and bulk water, respectively.[6a] In D_2O such conversion is not observed and there is a formation of HD in greater quantities than D_2. This indicates[25a] that, in contrast to platinum,[25b] the enzyme heterolytically splits the H_2 molecule into a hydron and a hydride, $H_2 \rightarrow H^+ + H^-$.

The HD/H_2 ratio in proton–deuterium (or proton–tritium) exchanges with water, for example, $H_2 + D_2O \rightarrow HD + HDO$, is pH dependent. It has therefore been assumed that during the heterolytic cleavage, the proton binds a base near the catalytic site.[6a]

Redox-poised states of the hydrogenase catalytic sites and iron–sulfur clusters and their correlation with enzymatic activities have been extensively studied by various spectroscopic methods, as discussed in several thorough reviews on the NiFe[6a, 24] and FeFe[2a, 2b] enzymes. The most recent works include electron paramagnetic resonance (EPR) and ^1H electron nuclear double resonance (ENDOR) spectroscopies,[26, 27a, 27b] Mössbauer spectroscopy,[27] vibrational (IR) spectroscopy,[16, 28] X-ray absorption studies,[29] and X-ray crystallography,[9b, 12, 17] possibly coupled with isotopic labeling and/or redox titrations[30] and/or theoretical calculations (see below).

EPR spectroscopy of NiFe hydrogenases with ^{61}Ni has identified three distinct signals, termed Ni-A, Ni-B, and Ni-C for historical reasons. The Ni-A and Ni-B signals are observed under aerobic conditions where the enzyme is catalytically inactive. Ni-A identifies a form of the enzyme (Ni-A unready) which requires hours of reductive treatment before the appearance of active enzyme whereas Ni-B identifies a form (Ni-B ready) which is readily activated, in the absence of O_2, upon addition of H_2. The Ni-C signal is observed when the enzyme is fully active. Upon illumination at 30 K, the Ni-C form yields a distinct EPR signal called Ni-L. Moreover, stoichiometric oxidative and reductive titrations monitored by EPR and Mössbauer spectroscopies have established that the reduction of the Ni-B form into the Ni-C form involves two successive one-electron steps.[30a] The one electron reduced Ni-B form is EPR silent (the so-called Ni-SI (for silent) form). Full reduction of the enzyme leads to the Ni-R (for reduced) form which is EPR silent and one electron more reduced than the Ni-C form. The Ni-A, Ni-B, Ni-C, and their respective one electron reduced EPR silent forms, Ni-SU (for silent unready), Ni-SIa and Ni-SIb (for silent intermediate), and Ni-R (for reduced) have all been characterized by vibrational spectroscopy. The two infrared-identified forms Ni-SIa and Ni-SIb most probably depend on the protonation state of the catalytic site.[28a] Moreover, EPR-coupled redox titrations show that the two [4Fe–4S] clusters of *D. gigas* hydrogenase also change redox states upon activation of the enzyme. Consequently, any redox state of this hydrogenase appears as a combination of the redox states of the catalytic site and the iron–sulfur clusters.[30b]

The formal oxidation states of the nickel atom of the NiFe hydrogenase dinuclear center during hydrogen activation have been the subject of intense investigation,[5, 6, 29c, 31, 32] as exemplified by some recent spectroscopic studies. On the one hand, nickel L-edge X-ray absorption and vibrational spectroscopy studies of several NiFe hydrogenases and model compounds in different conditions (for example, the oxidized or H_2- or dithionite-reduced forms)[29b] led to the proposal that the redox state of nickel is Ni^{III} in the Ni-A oxidized inactive form, as previously demonstrated,[6a, 26a] but high-spin Ni^{II} in the reduced active or CO-inhibited forms, which is contrary to many spectroscopic data and theoretical calculations (see below).[6, 26a, 28b] However, on the other hand, recent density functional theory (DFT) descriptions and calculations of the magnetic resonance parameters of the *D. vulgaris* NiFe hydrogenase show that a good agreement between the structural parameters and the experimental g-tensor values is only obtained when Ni-C is low-spin Ni^{III} with an NiFe bridging hydride.[26c, 26d] By contrast, it is now probable that the iron atom at the catalytic site is low-spin Fe^{II} in all the identified redox states of the enzyme as shown by EPR[26b] and ENDOR spectroscopies with ^{57}Fe-enriched enzyme.[26a]

Although to a lesser extent, several redox-poised states of FeFe hydrogenases and their correlation with the catalytic activity have also been been characterized by the same spectroscopic techniques used for the NiFe hydrogenases.[2a, 2b, 16d, 27b, 27c, 28c] For example, upon reductive activation of the aerobically prepared inactive *D. vulgaris* FeFe hydrogenase, a first rhombic EPR signal, termed $H_{ox\text{-}2.06}$, appears, which can be assigned to an oxidized form of the "H-cluster" (Figure 2). The signal then disappears as a second rhombic EPR signal, termed $H_{ox\text{-}2.10}$, is detected and disappears in turn. The latter $H_{ox\text{-}2.10}$ signal, detected for all FeFe hydrogenase studied so far, is not observed for the reduced active form of the enzymes.[2a, 27b, 27c] However, as for NiFe hydrogenases, the formal oxidation states of the iron atoms in the FeFe hydrogenase dinuclear center during this activation process, or catalysis, can still be discussed in the light of some recent spectroscopic studies. Analysis of Mössbauer spectroscopy data for the *C. pasteurianum* FeFe hydrogenase shows that the dinuclear center presumably contains two low-spin Fe^{II} sites in the reduced state and that the proximal [4Fe–4S] cluster remains in the formal 2^+ oxidation state in the oxidized inactive, CO-inhibited, and reduced states. On the basis of the latter observation it has been inferred that the dinuclear center is mixed valent Fe^{III}–Fe^{II} in the oxidized and CO-inhibited states and that the Fe^{III} site is probably the most distant iron atom from the $[4Fe\text{–}4S]_H$ cluster, that is, Fe2 (Figure 2).[27b] In this context, it is of interest to note that this same iron atom is the CO-binding[33] or the putative H_2-binding site (Figure 2).[13, 14, 22] However, in view of the as yet limited

knowledge on the electronic properties of the Fe—Fe dinuclear center, the authors did not exclude the possibility that the redox couple of the Fe—Fe dinuclear center could be actually Fe^{I}—Fe^{I}/Fe^{I}—Fe^{II} in accordance with model compound studies.[40a, 40b] A more recent analysis of a vibrational spectroscopy study of the *D. desulfuricans* FeFe hydrogenases shows that the dinuclear center is probably Fe^{II}—Fe^{I} in the oxidized and CO- and ^{13}CO-inhibited forms. The Fe^{I} state has been tentatively assigned to Fe2 (Figure 2) since, in particular, an Fe^{I} state could facilitate the donation of π-electron density from Fe2 to CO in the protein,[28c] again in accordance with model compound studies.[40a, 40b] A very recent Mössbauer spectroscopy study of the *D. vulgaris* hydrogenase, mentioned-above, came to similar conclusions, that is, that a low-spin Fe^{I}—Fe^{II} or Fe^{III}—Fe^{II} state exists for the oxidized enzyme.[27c] Moreover this same work suggests that the reductive activation begins with the reduction of the proximal $[4Fe—4S]_H$ cluster.

4. Model Compounds and Theoretical Calculations

The preparation of functional organometallic compounds which could be used, instead of platinum, as cheap catalysts for hydrogen production remains a remote goal.[34] However, the large body of biochemical, spectroscopic, and crystallographic data on NiFe[6a] and FeFe[2a, 2b] hydrogenases[5] has already been extensively used as a basis for the synthesis and spectroscopic analysis of "analogues" (also termed "model compounds") of the enzyme's catalytic sites or sections thereof. This approach has turned out to be a very powerful tool for improving our understanding of the enzyme's structural, spectroscopic, and/or catalytic properties while searching for synthetic pathways to new, possibly functional, catalysts. In parallel, theoretical studies based on density functional theory and spectroscopic/crystallographic data have contributed to the proposal of detailed schemes for the catalytic mechanisms.

Prior to the determination of the crystal structures, it was thought that the active site of NiFe hydrogenases consisted of a single nickel atom with thiolate protein ligands. Accordingly, organometallic chemistry studies focused on the synthesis and spectroscopic characterization of nickel thiolate complexes. These studies confirmed, in particular, the role of the thiolate ligands in accepting a proton (Brönsted base) and thereby assisting the heterolytic cleavage of molecular hydrogen. Since the discovery of the metallic dinuclear centers of NiFe and FeFe hydrogenases and their unprecedented nonproteic ligands, the major questions have focused on the possible role of the two metals and their cysteic sulfur or nonproteic ligands in the hydrogenase's catalytic activity. Along these lines, model chemistry of the active site of NiFe hydrogenases has headed for hydrogen activation by metal sulfides and the role and properties of the $Fe(CO)(CN)_x$ moiety(ies) (where $_x = 1, 2$). This field has been thoroughly discussed in a recent review.[35] Here we highlight some of the recent data.

Models of the $Fe(CO)(CN)_2$ moiety of NiFe hydrogenases have been synthesized and characterized by spectroscopy and electrochemistry.[36] Of particular interest is the trithiolate compound $[Fe^{II}(PS3)(CO)(CN)]^{2-}$ compound ($(PS3)H_3$ = tris(2-phenylthiolphosphine).[36b] Mössbauer spectroscopic analysis has established that the iron atom is low-spin Fe^{II}, and it has been suggested that an as yet unexplained small isomer shift observed in a Mössbauer spectrum of one NiFe hydrogenase[27a] should be assigned to the iron atom of the NiFe center. In addition, since the redox-induced shifts in the stretching frequencies of CO and CN^- of this same compound (approximately 100 cm^{-1}) are far larger than the redox-induced shifts of the enzyme (about 10 cm^{-1}) it has been inferred that the redox state of the iron atom at the catalytic site remains unchanged.[36a] Another model compound, $[(\eta^5\text{-}C_5H_5)Fe(CO)(CN)_2]^-K^+$ has been synthesized and found to mimic the structural and spectroscopic properties of the $Fe(CO)(CN)_2$ moiety of the enzyme active site; this also led to the conclusion that the six-electron, anionic (η^5-C_5H_5) moiety could mimic the donating ability of the nickel thiolate moiety, $Ni(\mu\text{-}SCys)_2(\mu\text{-}OH)$ in the NiFe enzyme active site. The latter result has prompted further systematic vibrational spectroscopy and electrochemical studies of $[(\eta^5\text{-}C_5H_5)Fe(CO)(CN)_2]^-$ salts to probe, in particular, the effects on CO and CN^- ligand vibrational modes of electronic changes (by replacing (η^5-C_5H_5) wth (η^5-C_5Me_5), alkylation, hydrogen bonding, or ion pairing to cyanide nitrogen) and Fe redox states.[37] Taken together, the vibrational spectroscopy studies on $[(\eta^5\text{-}C_5H_5)Fe(CO)(CN)_2]^-K^+$ model compounds and the NiFe enzymes mostly show that (1) the iron atom at the dinuclear center of NiFe hydrogenases is not redox active, (2) the CO and CN^- ligands are indeed "sensing" electron-density changes, (3) a hydrogen-bonding environment is correlated to the relative intensities of the ν(CO) and ν(CN) bands.

$^1H/^2H$ NMR and infrared spectroscopies of the reaction products of the nickel thiolate complex $[Ni(HNPnPr_3)('S_3')^{2-}]$ (where $(HNPnPr_3)$ = phosphorane imine and $('S_3')^{2-}$ = bis(2-sulfanylphenyl)sulfide(2−) with D_2 have shown that this compound, mimicking the nickel site moiety of NiFe hydrogenases, is able to catalyze H^+/D_2 exchange.[38a] A detailed mechanism of the reaction shows that D_2 is heterolytically cleaved with the assistance of Lewis acidic Ni^{II} and Brönsted basic thiolate sulfur donors. This suggests that H_2 heterolysis by active NiFe hydrogenases does not require a Ni^{II} reduction. In addition, it has been proposed that the distorted NiS_4 geometry observed in model compounds and in the enzyme nickel site as well might favor their interaction with H_2.[38a, 38b] Starting with the complex $[Fe(NS_3)(CO)]^{-6}$ as a chelate ligand to a nickel atom, a bis(thiolate-bridged) Ni – Fe complex containing one nickel atom and one iron atom bridged by two thiolate groups and with two CO ligands attached to the iron has been synthesized.[38c] Crystallography and infrared spectroscopy show similarities between this complex and the dinuclear active site of NiFe hydrogenases.[8, 9, 12] However, as in other thiolate-bridged NiFe compounds,[35] the NiFe distance (here 3.31 Å) is significantly longer than that observed in the oxidized or reduced form of the NiFe enzymes (2.9 and 2.6 Å, respectively); this points again at a bond-type interaction between the two metals, or delocalization, at the active site of the enzymes.

Prior to the determination of the FeFe hydrogenase crystal structures, the dinuclear iron compound $[Fe^{II}$ (dsdm)(bmes)Fe^{II}-

(CO_2)], containing bridging thiolate ligands and terminal CO ligands to one iron, was synthesized and characterized by vibrational and Mössbauer spectroscopy (dsdm = *N*,*N*′-dimethyl-*N*,*N*′-bis(2-sulfanylethyl)ethylene diamine; bmes = 2-bis(sulfanylethyl)sulfide).[39] The IR spectrum of this model compound shows strong bands, assigned to the carbonyl ligands, within the range observed for FeFe hydrogenases. It is of interest to note the Fe atom attached to the CO-ligands is low spin. Moreover the Fe—Fe distance is 0.31 nm, which excludes any metal bonding. Since then, several dinuclear iron–sulfur complexes of the type $[Fe_2(S_2C_3H_6)_2(CN)_2(CO)_4]^{2-}$, with CO and CN^- ligands on the iron atoms and a bridging propane dithiolate, have been prepared and characterized; these complexes mimic the structure and catalytical properties of the FeFe hydrogenases active site.[40] The match between the structures of the model compounds and those of the protein active site in their oxidized[13], partially or fully reduced,[14, 17] or CO-inhibited[18] forms is good. The largest discrepancies concern the Fe—Fe distances, which are slightly shorter in the model compounds (2.51–2.52 Å versus 2.6 Å in both proteins), and the presence of an Fe—Fe bridging CO ligand in the oxidized proteins. As in other iron complexes with CO or CN^- ligands, the Fe—CO distances are shorter than the Fe—CN distances. This could result from regioselective synthesis.[40b] However, a detailed stereochemical analysis of the crystal structures, along with earlier spectroscopic data (that is, Electron Spin Echo Envelope Modulation (ESEEM)),[2a, 41] has led to the suggestion that the molecule bridging the two iron atoms at the FeFe hydrogenases active site is in fact a di(thiomethyl)amine ($^-SCH_2NHCH_2S^-$)[17] and not a propane dithiolate as previously thought.[14] This bridging amine could be involved in a base-assisted heterolytic cleavage of hydrogen. The synthesis of new model compounds is greatly anticipated to clarify these points.

Theoretical calculations with DFT have been performed in conjunction with structural and spectroscopic data to investigate the structure of the redox-poised states of the catalytic site of the NiFe hydrogenases or their analogues.[42] In one case the protein "matrix" has been taken into account by combining DFT and a molecular mechanics energy function.[42c] These works have been compared and discussed in two recent reviews.[43] In short, they point to a heterolytic cleavage of the hydrogen molecule; this probably leads to the protonation of a cysteic sulfur ligand (for example, *D. gigas* *Sg*Cys 530; Figure 1b). The redox states of the forms putatively involved in the catalytic cycle were found to be Ni^{II}—Fe^{II}, with or without a bridging hydrogen atom for Ni-SIb, $Ni^{III,I}$—Fe^{II} with a bridging hydrogen atom for Ni-C and Ni^{II}—Fe^{II} with a bridging hydrogen atom for Ni-R. However the proposed catalytic schemes differ in their hydron location and the nickel redox state for Ni-C. It is noteworthy that in all schemes the iron atom is low-spin Fe^{II} and redox inactive.

5. Toward a Catalytic Mechanism of Hydrogenases?

The localization of the chemical reaction components, that is, hydrogen molecule, hydron, or hydride, in the NiFe and FeFe hydrogenases during the catalytic cycle remains elusive. However there is a consensus on a catalytic reaction occurring at a vacant, or potentially vacant, terminal coordination site of the nickel atom in NiFe hydrogenases and one of the two dinuclear center iron atoms (Fe2) in FeFe hydrogenases (Y in Figure 1b and 2). Both sites are indeed occupied by CO in the CO-inhibited enzymes and are close to one end of the hydrophobic channels which could mediate molecular hydrogen transfer between the catalytic site and the molecular surface.

In NiFe hydrogenases, three forms identified by EPR and/or IR seem to be involved in the catalytic cycle, Ni-SI, Ni-C, and Ni-R. H_2 most probably binds to an Ni-SI form (2+) and splits into a hydride and a hydron, which in turn binds to a nearby base (for example, *Sg*Cys 530 in *D. gigas*; Figure 1b). The $Fe(CO)(CN)_2$ moiety is not redox active and the iron atom is formally Fe^{II}. Therefore, the role of the iron atom might be merely binding a hydride during the catalytic cycle. There is also a consensus on the presence of a hydride bridging the two metal atoms in the Ni-C form.[6a, 26d, 29c, 43] Moreover, as recently pointed out for NiFe hydrogenases,[29c] spectroscopic EPR, X-ray absorption, and IR data of the enzyme's various redox states indicate an electronic delocalization over the entire active site, including the metals and the sulfur and CO/CN^- ligands. This means that the usual assignation of formal oxidation states to the metals does not reflect the charge density distribution.[26d]

In FeFe hydrogenases H_2 binds to Fe2 and splits into a hydride and a hydron which could bind to a nearby cysteic or Fe—Fe bridging sulfur atom. The redox-linked switch of a CO ligand from an Fe—Fe bridging position in the oxidized state to an Fe2 terminal position in the reduced one might reflect the electronic changes which occur upon Fe2 reduction or/and hydride binding.[17] Finally, it should be pointed out that the "role" of the sulfur, CO, and CN^- ligands is probably to reinforce the electrophilic character of the Ni—Fe or Fe—Fe dinuclear clusters; this facilitates reversible hydrogen binding and a subsequent base-assisted heterolytic cleavage.

A better understanding of the catalytic mechanism of NiFe and FeFe hydrogenases does obviously require more experimental and theoretical work, such as genetic engineering (see, for example, ref. [44]), spectroscopic studies (particularly single crystal ^1H ENDOR spectroscopy; see ref. [26d] and references therein), and high-resolution crystallographic studies of the anaerobically purified enzymes frozen in various redox states. This should help, for example, to understand why the activity and affinity for molecular hydrogen of NiFe and FeFe hydrogenases differ so much (Table 1). One of the most fascinating scientific challenges concerns the biosynthesis of the enzymes, particularly the assembly of their complex catalytic sites (Figure 1b and 2). Along these lines, extensive genetic studies of the maturation process of an NiFe hydrogenase from *Escherichia coli* have allowed the elucidation of several important steps in the incorporation of the nickel and iron atoms, which constitute the dinuclear core of the catalytic site, into the protein. More recently, it was discovered for the same NiFe hydrogenase that carbamoyl phosphate is specifically required for the synthesis of the catalytic site, probably as a source of the CO and CN ligands (see ref. [45] and references therein). Another exciting field has also been developed with the discovery that some NiFe hydrogenases act as hydrogen sensors in the regulation of the

hydrogenase genes but are not involved in hydrogen production or consumption.[46] Of course, it is difficult to predict how and when the identification of stable enzymes and/or synthesis of stable and functional model compounds, the central goals of hydrogenase research, will be achieved. However, the breakthroughs realized for the last few years by the hydrogenase research community[5, 35] have given a clearer picture on possible strategies.[48]

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[48] **Note added in proof:** Of great interest are some recent syntheses and studies of model compounds of FeFe hydrogenases performed by the groups of Rauchfuss (*J. Am. Chem. Soc.* **2001**, *123*, 9476 – 9477; *J. Am. Chem. Soc.* **2001**, *123*, 12518 – 12527; *J. Am. Chem. Soc.* **2002**, *124*, 726 – 727) and Darensbourg (*J. Am. Chem. Soc.* **2001**, *123*, 9710 – 9711). Moreover, the fidelity of nickel incorporation into the large subunit has been shown by the Böck group to depend on an active-uptake system that delivers nickel to the intracellular maturation machinery (*FEBS Lett.* **2001**, *499*, 73 – 76).

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