DOI: 10.1002/ejic.201000955

# Structure and Function of [Fe]-Hydrogenase and its Iron–Guanylylpyridinol (FeGP) Cofactor

**Seigo Shima*[a,b] and Ulrich Ermler[c]**

**Keywords:** Bioinorganic chemistry / Hydrogenases / Structure elucidation / Oxidoreductases / Structure–activity relationships

[Fe]-hydrogenase functions in the methanogenic pathway of hydrogenotrophic methanogenic archaea. It catalyzes the reversible reduction of methenyltetrahydromethanopterin (methenyl-$H_4MPT^+$) with $H_2$ to methylene-$H_4MPT$ and a proton by transferring a hydride ion to the *proR* position of the C14a carbon atom of methylene-$H_4MPT$. This third type of hydrogenase contains a unique iron–guanylylpyridinol (FeGP) cofactor, in which the iron atom is ligated by one cysteine sulfur atom, two CO groups, one solvent molecule, and an $sp^2$-hybridized nitrogen atom and an acyl carbon atom from the pyridinol ring. Three globular folding units of this protein form two clefts that serve as substrate-binding and active sites and that can be open or closed. Structural data are presented for the apoenzyme in a closed form, the holoenzyme (enzyme with the FeGP cofactor), the C176A holoenzyme, and the binary C176A holoenzyme-methylene-$H_4MPT$ complex in an open form. A closed and potentially active binary complex has been reliably modeled on the basis of the open binary complex and the closed apoenzyme. In this model, the iron center sits near the *Re* face of the imidazolidine ring of the substrate. The iron ligation site *trans* to the acyl carbon atom is next to the C14a carbon atom and is therefore considered to be the $H_2$ binding site.

## Introduction

Hydrogenases reversibly catalyze the heterolytic cleavage of $H_2$.[1,2] In the reverse reaction, $H_2$ is produced from the electron/hydride donor and proton(s). Three types of hydrogenases are known.[3] [NiFe]-hydrogenases and [FeFe]-hydrogenases contain dinuclear metal catalytic centers composed of nickel–iron and iron–iron ions, respectively.[2] The third type of hydrogenases, [Fe]-hydrogenases, contains a monatomic iron catalytic center.[3]

The systematic name of [Fe]-hydrogenase is $H_2$-forming methylenetetrahydromethanopterin (methylene-$H_4MPT$) dehydrogenase (Hmd). The enzyme catalyzes the reversible hydride transfer from $H_2$ to methenyltetrahydromethanopterin (methenyl-$H_4MPT^+$) (Figure 1).[3,4] The products of this reaction (and the substrates of the reverse reaction) are methylene-$H_4MPT$ and a proton. This enzyme is found in

[a] Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße, 35043 Marburg, Germany
Fax: +49-6421-178109
E-mail: shima@mpi-marburg.mpg.de
[b] PRESTO, Japan Science and Technology Agency (JST), Honcho, Kawaguchi, Saitama 332-0012, Japan
[c] Max Planck Institute for Biophysics, Max-von-Laue-Straße 3, 60438 Frankfurt/Main, Germany

*Seigo Shima was born in 1960 in Osaka, Japan. He studied agricultural chemistry at Osaka Prefecture University in Japan, where he received his B.S. degree in 1983 and MS degree in 1985. He then worked as a researcher in the Central Research Institute for Electric Power Industry in Chiba, Japan from 1985 to 1993. He obtained his Ph.D. in 1991 from the University of Tokyo. In 1993, he was awarded a fellowship from Alexander von Humboldt Foundation to join the group of Rudolf K. Thauer at the Philipps-University in Marburg, Germany. In 1995, he was appointed as a group leader at the Max-Planck-Institute for Terrestrial Microbiology in Marburg. He is also a PRESTO researcher of Japan Science and Technology Agency (JST) from 2009. His current research interests focus on the catalytic mechanism of [Fe]-hydrogenase and the biosynthesis of the FeGP-cofactor and the enzymes involved in methanogenesis and anaerobic oxidation of methane.*

*Ulrich Ermler was born in 1958 in Weingarten/Württemberg, Germany. He studied chemistry at the University of Freiburg (1978–1985) and completed his Ph.D. in 1990 working with Georg Schulz at the Institute of Organic Chemistry and Biochemistry. In 1990–1991, he worked as postdoctoral scientist in the group of Gunter Schneider at the Biomedical Center in Uppsala, Sweden. He then joined the group of Hartmut Michel at the Max-Planck-Institute of Biophysics in Frankfurt, Germany, where he is a group leader since 1995. His research interests are directed toward the structure and mechanism of diverse metal- and coenzyme-dependent proteins with the focus on enzymes of methanogenesis.*

  

many hydrogenotrophic methanogenic archaea and catalyzes one reaction step in the methanogenic energy-conserving pathway from $CO_2$ and $H_2$.[5] [Fe]-hydrogenase does not contain a Fe–S cluster but instead a unique iron guanylylpyridinol (FeGP) cofactor (Figure 2), which can be extracted from [Fe]-hydrogenase. The recent crystallographic, spectroscopic, and chemical analyses of [Fe]-hydrogenase reveals that its iron center is ligated by one cysteine sulfur atom, two CO groups, one solvent molecule, and an $sp^2$-hybridized pyridinol nitrogen atom and an acyl carbon atom of the substituent of the pyridinol ring. The structure of the FeGP cofactor is described in detail below.[6–10]

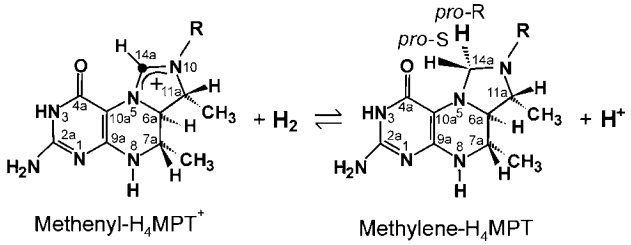


Figure 1. The reaction catalyzed by [Fe]-hydrogenase ($H_2$-forming methylenetetrahydromethanopterin dehydrogenase, Hmd). A hydride is reversibly transferred from $H_2$ to the *proR* position of methenyl-$H_4$MPT$^+$.[4,11]

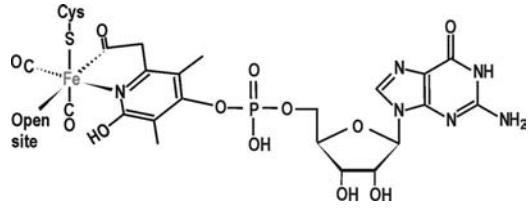


Figure 2. FeGP cofactor of [Fe]-hydrogenase.[7,10] The position of the intrinsic CO group arranged *trans* to the Cys176-sulfur atom has been determined by X-ray crystallography for both wild-type and C176A holoenzymes. The second intrinsic CO-binding-site *trans* to the pyridinol nitrogen atom has been identified in the crystal structure of the C176A holoenzyme, but cannot be unambiguously assigned in the wild-type holoenzyme. The open site *trans* to the acyl-carbon ligand appears to bind a solvent molecule, which is proposed to be removed in the catalytic cycle.[12,13] The open site could also be used as an alternative binding site for the second CO ligand.[7]

[Fe]-hydrogenase catalyzes a single and double exchange between $D_2$[14–16] and protons of bulk water and a conversion of *para*-$H_2$ into *ortho*-$H_2$, both only in the presence of methenyl-$H_4$MPT$^+$.[17] Thus, $H_2$ activation necessarily requires the substrate. [Fe]-hydrogenase also catalyzes a stereospecific direct exchange of the *proR* hydrogen of methylene-$H_4$MPT with protons of water[18] but does not reduce artificial dyes or one- or two-electron acceptors, neither in the absence nor in the presence of methenyl-$H_4$MPT$^+$.[4] Thus, [Fe]-hydrogenase specifically reduces methenyl-$H_4$MPT$^+$.

Crystal structures of the apoenzyme (without FeGP cofactor and the substrate) and holoenzyme (with FeGP cofactor but without substrate) of [Fe]-hydrogenase have been reported. Recently, the crystal structure of the binary holoenzyme–substrate complex of [Fe]-hydrogenase was elucidated and provides valuable information about the catalytic mechanism. In this review, we focus on the structural analysis of the [Fe]-hydrogenase, the FeGP cofactor, and the proposed catalytic mechanism involving the open and closed enzyme forms.

## Structure Analysis of the FeGP Cofactor

Buurman et al.[19] found that [Fe]-hydrogenase contains a compound – later termed FeGP cofactor – with a molecular mass of ca. 700 Da. The size suggests a rather bulky organic proportion. Mass spectrometry of the isolated cofactor indicates the presence of iron, which has been confirmed by chemical analysis.[20] The FeGP cofactor can be extracted from [Fe]-hydrogenase, and the fully active holoenzyme can be reconstituted from the isolated cofactor and the apoenzyme heterologously produced in *Escherichia coli*.[19,20]

The structure of the iron complex in the FeGP cofactor was mainly analyzed by using spectroscopic methods. Mössbauer and infrared spectroscopic data reveal that the cofactor contains a single iron species; the infrared data also indicate two intrinsic CO ligands of the iron site at an angle of 90°. According to X-ray absorption spectroscopic data, a sulfur-containing compound ligates to the iron of the protein-free and -bound FeGP cofactor. The thiol ligand of the enzyme is provided by Cys176 (*Methanocaldococcus jannaschii* nomenclature), as deduced from a mutational analysis of the conserved cysteine residues. The iron of the protein-free FeGP cofactor appears to be complexed with 2-mercaptoethanol, which was added for cofactor stabilization.

According to the earlier Mössbauer spectroscopic data, the iron atom in the FeGP cofactor could be either in a low-spin $Fe^0$ or in a $Fe^{II}$ oxidation state.[21] Later studies support the iron in the low-spin $Fe^{II}$ state on the basis of related infrared and Mössbauer data of the protein-free (-bound) FeGP cofactor and monoiron model complexes,[22] related X-ray absorption spectra of [Fe]-hydrogenase and model compounds,[23] and the occupation of the iron site of the C176A structure with six ligands.[7,24]

The structure of the organic part of the FeGP cofactor could not be analyzed directly by NMR spectroscopy and mass spectrometry because the cofactor is unstable under heat and in light. The UV and blue light (<500 nm) absorbed by the FeGP cofactor rapidly decomposes the cofactor (half life of 15 min under white light with a photon fluence rate of 2600 $\mu mol\,m^{-2}\,s^{-1}$ at 4 °C). Infrared spectroscopic analysis reveals that the intrinsic CO ligands are released from the iron site upon illumination, and this might cause a conversion of low-spin iron to a high-spin paramagnetic species. After isolation of the FeGP cofactor, the solution always contains some light-decomposed paramagnetic species that interfere with the NMR spectroscopic analysis.

Therefore, structural information on the organic part was obtained by using isolated cofactor fully decomposed by

  

light and separated from the released iron, CO, and 2-mercaptoethanol (Figure 3). NMR spectroscopy and mass spectrometry of the decomposed organic part reveals a guanylylpyridone derivative, (6-carboxymethyl-3,5-dimethyl-2-pyridone-4-yl)-(5′-guanosyl) phosphate, in which the pyridone ring is linked with the guanosine base by a phosphodiester bond. This structure suggests a possible iron coordination of the pyridone nitrogen atom and of the carboxyl oxygen atom of the carboxymethyl substituent to yield a six-membered ring.[25] It is important to note that since the guanylylpyridinol compound is formed from the active FeGP cofactor by light inactivation, the identified structure might deviate from the original structure.[25] The most recently proposed model of the FeGP cofactor contains an acyl rather than a carboxylate ligand as the iron ligand (see below).

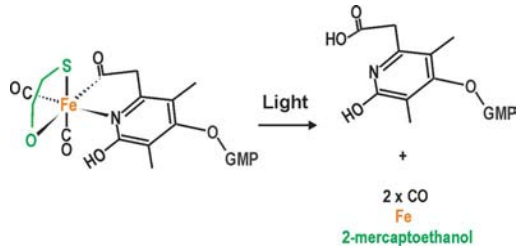


Figure 3. The formation of the decomposed FeGP cofactor. The iron-ligation structure of the protein-free FeGP cofactor was estimated from the C176A holoenzyme structure.[7] The FeGP cofactor isolated from the [Fe]-hydrogenase in the presence of 2-mercaptoethanol was decomposed by irradiation with light. The guanylylpyridinol part was purified from CO, iron, 2-mercaptoethanol, and the non-decomposed cofactor and its structure was analyzed by NMR spectroscopy and mass spectrometry.

The cumulative data of the structure analysis provides a fairly clear picture of the composition of the iron complex of the FeGP cofactor. However, the detailed geometric information necessary for building a three-dimensional model is obtained from the crystal structure of the [Fe]-hydrogenase holoenzyme.

## Crystal Structures of the Apoenzymes

The crystal structures of the apoenzymes of [Fe]-hydrogenase from *Methanocaldococcus jannaschii* and *Methanopyrus kandleri* heterologously produced in *Escherichia coli* have been reported (Figure 4).[26] The crystal structure of the apoenzyme from *M. kandleri* was solved at 2.8 Å resolution by using the multiple anomalous dispersion method and a selenomethionine-labeled protein, but, for unknown reasons, the model could not be refined to a reasonable *R*-factor. The crystals of [Fe]-hydrogenase apoenzyme from *M. jannaschii* obtained in the presence of 1 mM dithiothreitol (DTT) diffracted to a resolution of 1.95 Å, and the phase problem was solved by using the molecular replacement method and the apoenzyme structure of *M. kandleri* as a search model. The refinement of the apoenzyme from *M. jannaschi* converges to $R/R_{free}$ factors of 19.1%/21.7%.

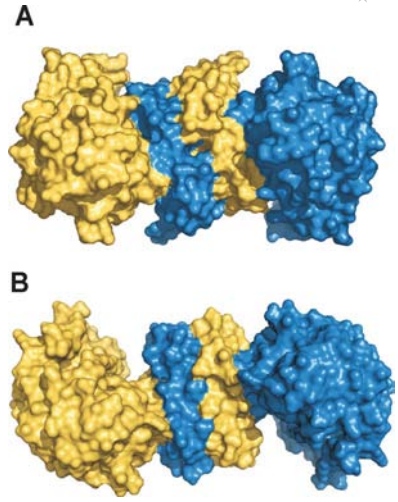


Figure 4. Crystal structure of the apoenzyme from (A) *Methanococcus jannaschii* (2B0J) and (B) *Methanopyrus kandleri*. The surface models of the homodimeric structures are shown. One monomer is indicated in yellow, the other in blue.

The homodimeric protein consists of two N-terminal peripheral units and one central globular unit, the latter composed of the C-terminal segments of both subunits. The N-terminal units represent a Rossmann fold, which is frequently used as a dinucleotide-binding domain. A cleft is formed between each of the peripheral and central folding units of the homodimer. The helical C-terminal segments of the two subunits are intertwined such that each N-terminal unit faces the C-terminal segment of the partner subunit. The structure of the N-terminal unit is most related to that of the NADP-dependent human L-3-hydroxyacyl-CoA dehydrogenase (Hdh; 2HDH). By superimposing the N-terminal unit of [Fe]-hydrogenase onto the Hdh-NADP complex, the guanosine monophosphate part of the FeGP cofactor could reasonably be modeled into the mononucleotide-binding site of the N-terminal unit. Interestingly, the apoenzyme from *M. kandleri* was crystallized in the open form, whose cleft size is larger than that of *M. jannaschii* in the closed form. This finding suggests that large conformational changes of the active-site cleft might take place during the catalytic reaction (see below).[26]

## Crystal Structures of the Holoenzyme

### FeGP Cofactor Model with a Carboxymethyl Group

The crystal structure of the [Fe]-hydrogenase holoenzyme reconstituted from the FeGP cofactor and the apoenzyme from *M. jannaschii* heterologously produced in *E. coli* has been reported.[10] Unexpectedly, the holoenzyme structure is found in the open conformation, although the apoenzyme from *M. jannaschii* is found in the closed conformation. Binding of the FeGP cofactor induces only minor conformational changes of a few residues of the N-terminal unit despite the open/closed transition.

  

The 1.75-Å structure reveals a large residual electron density at the mononucleotide binding sites of the N-terminal units (Figure 5),[10] which properly accommodates the organic part of the FeGP cofactor. As described above, the structure of the organic part was already known from NMR analysis of the light-decomposed FeGP cofactor. The binding mode of GMP to the protein essentially corresponds to that predicted on the basis of the apoenzyme structure. An iron atom was fitted into the highest electron density positioned in the plane of the pyridinol ring 2.1 Å apart from the pyridinol nitrogen atom, which indicates that the pyridinol nitrogen atom is $sp^2$ hybridized and that the ring is in the pyridinol rather than in the pyridone tautomeric form (Figure 6A). The binding strength between the $sp^2$-hybridized pyridinol nitrogen atom and the iron atom might be increased by a π back-bond, especially when the hydroxy group is deprotonated. The electron density clearly indicates that the iron center is coordinated to the Cys176-thiol group and an intrinsic CO ligand; the two ligands are positioned *trans* to each other.

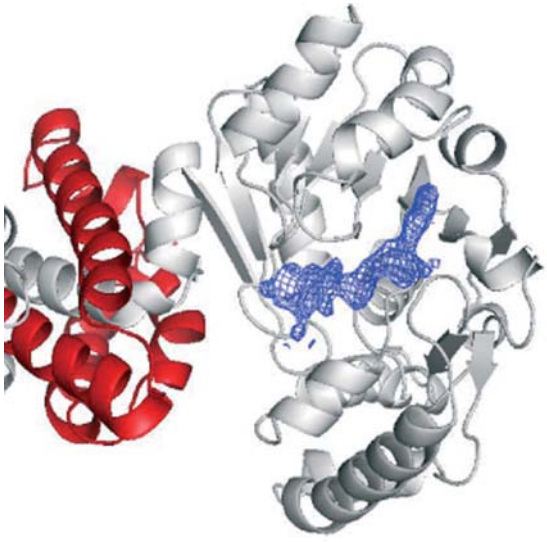

Figure 5. Ribbon diagram of the [Fe]-hydrogenase holoenzyme from *M. jannaschii* in the open form. One subunit is shown in white, the other in red. A residual electron density corresponding to the FeGP cofactor (depicted as a blue net) is attached to the peripheral unit, which contains a Rossmann fold.[10,26]

In a first attempt at fitting the data, the carboxymethyl group was positioned at the iron site *cis* to the pyridinol nitrogen atom (and *trans* to the unknown ligand, as shown in Figure 6A) because the electron density between the iron atom and the pyridinol ring is somehow connected. However, the electron density at this position does not match that of the bulky carboxymethyl group and could be reasonably interpreted as the second intrinsic CO ligand. Consequently, the pyridinol ring was turned and the carboxymethyl substituent of the pyridinol ring was modeled at the opposite side of the iron site, as shown in Figure 6A, even though the electron density is only visible up to the methyl moiety. The absent electron density at the solvent-exposed

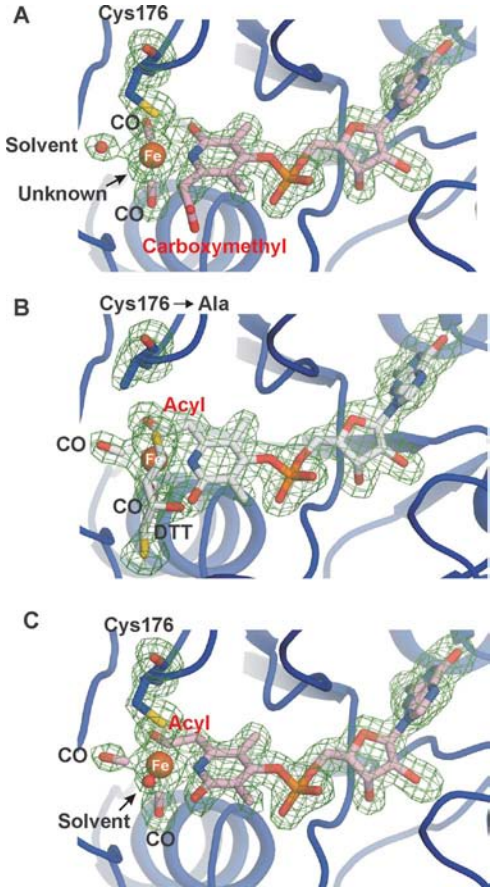


Figure 6. The iron center of the FeGP cofactor in the [Fe]-hydrogenase holoenzyme. (A) The initial model with a solvent-exposed carboxymethyl substituent of the pyridinol ring (3DAG). (B) The reinterpreted model containing the acyl-carbon ligand in the C176A holoenzyme (3F46). (C) The reinterpreted model containing the acyl-carbon ligand in the wild-type holoenzyme (3F47). The electron density maps around the iron complex are shown in green.

carboxyl group has been explained by the flexibility of the carboxyl group, which is believed to become fixed upon substrate binding.

The electron density *trans* to the second CO ligand could not be interpreted, and the ligand is therefore unknown. The ligation site *trans* to the pyridinol nitrogen atom of the potentially octahedral iron center is modeled as a solvent molecule hydrogen-bonded to another solvent molecule.[10] The center of the spherical electron density is ca. 2.7 Å apart from the iron center, which suggests a weak association.

On summarizing the data, the electron-density map around the iron center of the FeGP cofactor is interpreted such that the iron ion is square-pyramidally complexed with the Cys176-sulfur atom, two CO ligands, an $sp^2$-hybridized nitrogen atom of pyridinol, and an unknown ligand (Figure 6A). In this model, the hydroxy and carboxymethyl substituents are not bound to the iron ion. Notably, some pyridone–metal complexes are known to fix the metal by bridging the metal with the pyridone nitrogen atom and the hydroxy group.[27]

  

### FeGP Cofactor Model with an Acyl-Carbon Ligand

One year after the publication of the initial model,[10] the iron-ligation structure was revised on the basis of the crystal structure of C176A [Fe]-hydrogenase, where the sulfur atom of Cys176 ligated to the active-site iron center is replaced by an alanine group. This mutant enzyme is still able to bind the FeGP cofactor but is completely inactive.[7] To stabilize the iron complex, the crystallization solution contains 1 mM DTT. The overall structure of the C176A holoenzyme is almost identical to that of the wild-type holoenzyme in the open form. The root-mean-square deviation between the C176A and wild-type holoenzymes is 0.19 Å. However, the 1.95-Å electron-density map around the iron center of the FeGP cofactor led to a reinterpretation of the structure of the iron site (Figure 6B).

In the C176A holoenzyme structure, a DTT molecule is bidentately coordinated to the iron center and captures the space occupied by the carboxymethyl substituent in the initial model. A new model was generated by rotating the pyridinol ring by 180°. The carboxymethyl moiety lies in the electron density so far occupied by the hydroxy group at the shielded side of the cofactor. As already mentioned above, the size and profile of the electron density does not match that of the carboxymethyl group. To ensure an agreement between the model and the electron density, one of the oxygen atoms of the carboxyl group was removed from the model and the remaining acyl group was positioned into the ligation site of the second intrinsic CO ligand. At the ligation site *trans* to the pyridinol nitrogen atom, the electron density of the C176A holoenzyme fits better to that of a diatomic species than to that of a monatomic species, which is found in the wild-type holoenzyme (see above). The second CO ligand was therefore modeled to this site. Thus, in the C176A holoenzyme, the iron is hexacoordinated by two CO ligands, the $sp^2$-hybridized pyridinol nitrogen atom, the acyl carbon atom from the pyridinol substituent, and a hydroxy and a thiol group of DTT (Figure 6B).

On the basis of this result, the iron complex structure in the wild-type holoenzyme was reinterpreted (Figure 6C). A better fit is obtained when the pyridinol moiety chelates the iron with two rather than with one ligand, the $sp^2$-hybridized nitrogen atom and the acyl carbon atom, as found in the C176A holoenzyme structure (Figure 6B, C). The ligation positions of the pyridinol nitrogen atom, the Cys176-sulfur atom, and the intrinsic CO ligand arranged *trans* to the Cys176-sulfur atom are unchanged in the new model. The second intrinsic CO ligand might be positioned as in the C176A holoenzyme, *trans* to the pyridinol nitrogen atom, where initially a solvent molecule was modeled. Since the electron density at this position is spherical rather than diatomic (Figure 6C), this assignment must be considered with caution. The new model is compatible with the previously obtained X-ray absorption spectroscopic data, in which the incorporation of the carbon from the acyl group improved the fit index.[7] On the basis of a comparison between experimental and simulated X-ray absorption near edge spectra, the electronic structure of the iron complex, and therefore its catalytic property, appears to be essentially determined by the acyl group of the cofactor.[23] But further studies with chemical methods are required for a final assignment of the acyl ligand.

Several model compounds of the FeGP cofactor containing an acyl-carbon ligand have been synthesized.[22,28–31] For example, the geometry of the iron site of $[Ph_2PC_6H_4C(O)]Fe(SPh)(CO)_3$ and $[\{Ph_2PC_6H_4C(O)\}Fe(SPh)(CN)(CO)_2]^-$ is similar to that found in the proposed structure of CO- and cyanide-inhibited [Fe]-hydrogenase, respectively.[31] Recently, two model iron compounds of the FeGP cofactor containing a five-membered metallacycle with an acyl ligand were reported.[32,33] The infrared frequency of the model compounds is almost identical to those of the enzymes. These results strongly support the existence of the acyl-carbon–iron ligand in the FeGP cofactor. It is important to note that a stable σ-bonding metal–carbon ligand is rare in biological systems. The only known examples are methylcobalamine and adenosylcobalamin (coenzyme B12). In the catalytic reaction of acetyl-coenzyme A synthase and methyl-coenzyme M reductase, acetyl-nickel and methyl-nickel intermediates are discussed, but only as transient species of the catalytic reactions. The acyl-carbon–Fe bond in the FeGP cofactor, which exhibits a strong σ-bonding rather than a π-acid character, is the next example. The function and biosynthetic route of such a unique biological metal ligand is intriguing.

## Crystal Structure of the Holoenzyme–Substrate Binary Complex

To understand the reaction of [Fe]-hydrogenase on a molecular basis, information on the binding site and the conformation of the substrates, methenyl-$H_4MPT^+$ or methylene-$H_4MPT$, is indispensable because $H_2$ activation is dependent on their presence. Recently, the crystal structure at 2.15-Å resolution of the mutant C176A [Fe]-hydrogenase from *M. jannaschii* complexed with methylene-$H_4MPT$ has been reported.[12] This structure reveals the substrate binding mode and provides new insights into the catalytic mechanism of [Fe]-hydrogenase.

### The Strategy of Co-Crystallization

The reaction catalyzed by [Fe]-hydrogenase is reversible ($\Delta G^{\circ\prime} = -5.5\ kJ\,mol^{-1}$) (Figure 1), and the ratio of methenyl-$H_4MPT^+$ and methylene-$H_4MPT$ in the reaction mixture is dependent on the $H^+$ and $H_2$ concentrations.[34] During co-crystallization of active [Fe]-hydrogenase with the substrate, the enzymatic reaction proceeds in the glove box atmosphere of $N_2/H_2$ (95:5, v/v), which results in mixtures of methenyl-$H_4MPT^+$ and methylene-$H_4MPT$ in the crystallization solutions. Such heterogeneous conditions may hamper crystallization of the holoenzyme–substrate complex. The use of C176A holoenzyme for co-crystallization attempts can avoid such undesirable substrate mix-

  

tures because it is inactive but can still bind the cofactor and the substrate.[8] On the basis of this premise, the C176A holoenzyme of *M. jannaschii*, reconstituted with FeGP cofactor, was crystallized in the presence of methylene-$H_4$MPT[12] under conditions similar to those used with the wild-type[10] and C176A holoenzymes.[7]

### Overall Structure

The overall structure of the binary C176A-holoenzyme–substrate complex is highly similar to those of the C176A and wild-type holoenzymes, which is reflected in the root-mean-square deviations of 0.33 and 0.28 Å, respectively, by using 344 equiv. $C_\alpha$ atoms.[12] The conformation of the binary complex indicates an open form, which is similar to the wild-type and the C176A holoenzymes without substrate (Figure 7). The structure of the binary complex reveals a highly occupied FeGP cofactor in the established position including the iron ligation structure with the acyl ligand as described above.

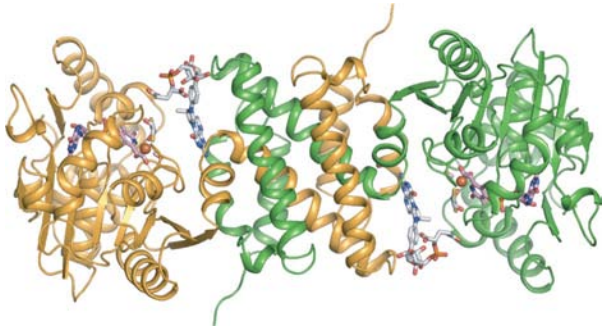

Figure 7. Ribbon diagram of the C176A [Fe]-hydrogenase holoenzyme from *M. jannaschii* in the complex with methylene-$H_4$MPT in an open form. The FeGP cofactor and methylene-$H_4$MPT (both depicted as color-coded stick models with the carbon atoms in pink and in gray, respectively) are attached to the peripheral and central units, respectively. The *Re* face of methylene-$H_4$MPT points towards the iron center of the FeGP cofactor. The distance between the C14a atom and the iron ion of the FeGP cofactor is 9.3 Å. The figure was modified from that by Hiromoto et al.[12]

### Methylene-$H_4$MPT Binding Site

Methylene-$H_4$MPT is clearly visible in the active site cleft as residual electron density that accurately corresponds to the shape of the pterin and imidazolidine rings (head part) and the phenyl ring (Figure 8). The occupancy of the head part is almost 100%. The head part and the phenyl ring of methylene-$H_4$MPT are arranged in a relatively extended conformation. The tail part is exposed to bulk solvent and is disordered.[12]

The extended conformation of methylene-$H_4$MPT found in the crystal structure of the C176A-holoenzyme–substrate complex definitely differs from the bent structure in solution determined by NMR spectroscopy (Figure 9)[35] and in the crystal structure of the formaldehyde-activating enzyme complex, in which the imidazolidine ring is kinked by 70° (not shown).[36] In contrast, the overall conformation of the head part and the phenyl ring of methylene-$H_4$MPT resembles that bound to [Fe]-hydrogenase from *Methanothermobacter marburgensis*, previously determined by NMR spectroscopy.[35,37] A closer look, however, reveals that the N5 and N10 atoms in the crystal structure are virtually in a planar conformation at N10, perhaps stabilized by π-bonding with the phenyl ring. In the NMR structure, N5 and N10 are in a tetrahedral $sp^3$-hybridized form, in which the lone pair of electrons of N5 and N10 lie on the *proR* and *proS* side, respectively.[35,37] It should be noted that the quality of the crystallographic data are at the resolution limit to distinguish between planar and nonplanar conformations at N5 and N10, especially when inaccuracies in the calculation of the geometric parameters for such a complex ring system used for refinement are considered. Therefore, the structure was carefully refined by using different parameter sets and analyzed on the basis of omit maps.[12]

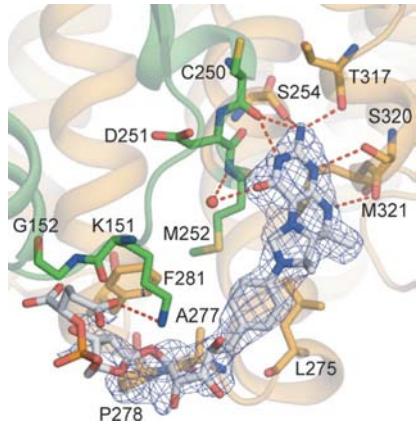


Figure 8. Methylene-$H_4$MPT structure and its interactions with the C176A [Fe]-hydrogenase. The $F_o - F_c$ omit electron-density map around methylene-$H_4$MPT (carbon atoms shown in gray) is contoured at the 2.8 σ level (in blue). The amino acid residues within 4 Å of methylene-$H_4$MPT are depicted as color-coded sticks. The peripheral unit including the hinge region (of one subunit) and the central unit (of the other subunit) are shown in green and orange, respectively. Hydrogen bonds and polar interactions are illustrated as broken lines. The figure was modified from that by Hiromoto et al.[12]

The reason for the difference in the N5 and N10 conformations might be that methylene-$H_4$MPT in the crystal structure is not present in an activated form. The activated conformation might be induced by interactions between the *Re* side of the imidazolidine ring and the Fe complex that cannot be formed in the open conformation. The planar-to-nonplanar transition of N5 and N10 might be caused by a rotation of the phenyl ring of methylene-$H_4$MPT upon cleft closure (see below). This hypothesis was inspired by Bartoschek et al., who proposed that the rotation angle of the phenyl ring could determine the side of the lone electron pair on the N10 atom.[35,37]

  

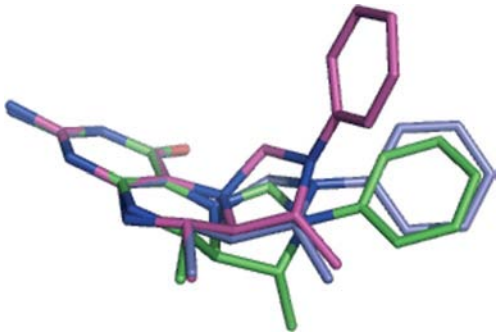

Figure 9. Conformations of methylene-$H_4$MPT. The methylene-$H_4$MPT structure of the C176A holoenzyme–substrate complex determined by X-ray crystallography (carbon atoms shown in green) [12] and the structure of methylene-$H_4$MPT in solution (carbon atoms shown in pink) and bound to the wild-type holoenzyme–substrate complex of *Methanothermobacter marburgensis* determined by NMR (carbon atoms shown in blue)[35,37] are superimposed and depicted as color-coded stick models. The tail part after the phenyl ring is omitted. The figure was modified from that by Hiromoto et al.[12]

Berkessel and Thauer proposed that inversion of the lone electron pair on the N10 atom and thereby activation of the *proR* hydrogen might be facilitated by protonating the lone electron pair of the N5 and N10 atoms by carboxyl groups.[38,39] However, no carboxyl group is present in the vicinity of the imidazolidine ring in either the open or closed form. The only proton donor nearby in the modeled closed form (see below) is the hydroxy group of the pyridinol ring, which could interact with the N10 atom of methylene-$H_4$MPT. With respect to the stereochemistry at C14a, this interaction would, however, be from the wrong side.[35] It could therefore be that the interaction between the hydroxy group and the N10 atom of methylene-$H_4$MPT actually mainly modifies the properties of the pyridinol. Such a "trigger" function of the substrate to activate the FeGP cofactor has been postulated by Yang and Hall.[13,40]

### Modeling of the Closed Form of the Binary Complex

The structure of the binary C176A-holoenzyme–methylene-$H_4$MPT complex reveals an open cleft between the central and peripheral units and a distance of 9.3 Å between the iron atom and the C14a atom of the substrate. This distance is obviously too long for transferring a hydride ion, even when $H_2$ binds in an end-on conformation. To model a catalytically productive conformation, we used the apoenzyme of [Fe]-hydrogenase from *M. jannaschii*, which was crystallized in the closed form.[26] The structures of the peripheral and central units of the closed apoenzyme and of the open C176A-holoenzyme–substrate complex are almost identical, as indicated by root-mean-square deviations of 0.58 and 0.42 Å, respectively, which implicate a large-scale rigid-body movement between the two globular units. Therefore, the closed form was modeled by superimposing the peripheral unit (1–241) and the central unit (253–345) of the binary complex onto those of the apoenzyme.[12] The movement of the peripheral unit from the open to the closed form corresponds essentially to a rotation of 35° (Figure 10 A, B). An analysis of the hinge region of the open and closed forms of [Fe]-hydrogenase suggests a

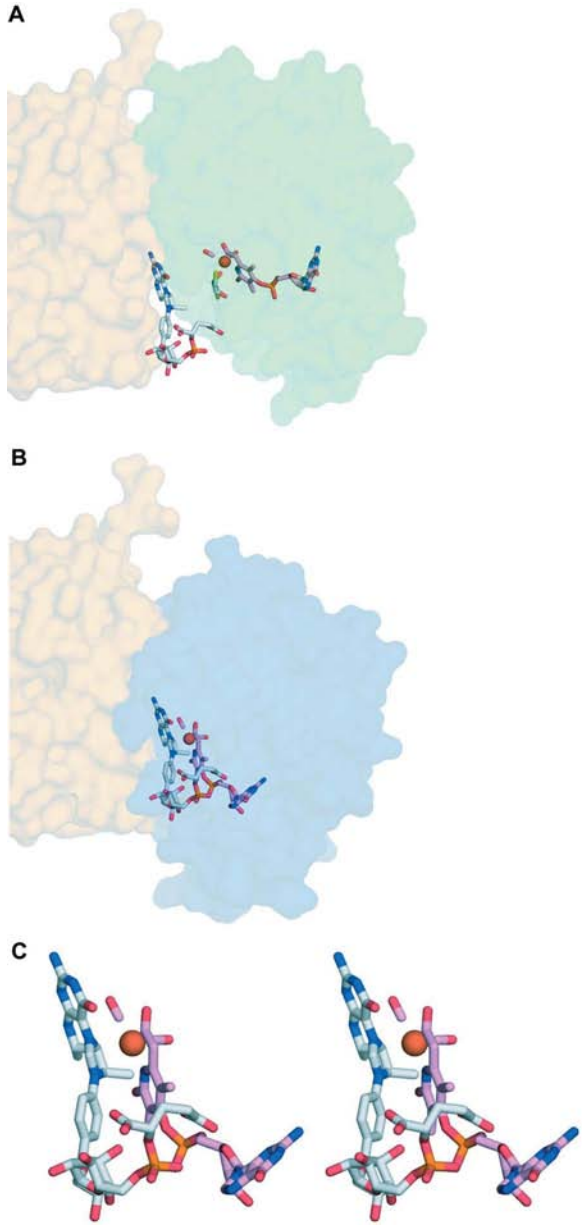


Figure 10. The conformational forms of [Fe]-hydrogenase.[12] Molecular surface representation (A) of the structure of the active-site cleft of the binary complex in the open form as experimentally determined and (B) of the modeled structure containing an active-site cleft in the closed form (see text). To prepare the model, the peripheral unit (green) and the central unit (orange) of the binary complex were superimposed onto the apoenzyme determined in the closed form,[26] which results in a rotation of 35° of the peripheral unit (blue) towards the central unit. The FeGP cofactor and methylene-$H_4$MPT are depicted as color-coded stick models with the carbon atoms in pink and in gray, respectively. DTT carbon atoms are shown in gray. In the closed model, DTT is omitted. (C) Close-up stereo view of methylene-$H_4$MPT and the FeGP cofactor in the closed form model. The distance between the C14a carbon atom and the iron ion is 3.0 Å in the closed model.

  

mechanism of how cleft closure upon substrate binding might be triggered.[12]

In the modeled closed form of the binary complex, new interfaces between the peripheral and central units are created. Besides DTT, only a few atoms of the FeGP cofactor coincide with methylene-$H_4$MPT, which indicates that the model of the closed form is reasonable but not optimal. Such possible orbital overlap would disappear upon conformational change of methylene-$H_4$MPT and/or the protein in the real closed form. Interactions between the FeGP cofactor and methylene-$H_4$MPT occur between the pyridinol and phenyl rings, between the hydroxyl group and the N10 atom, as well as between the CO ligand and the C4a oxo group (the latter coincide) (Figure 10C).

In the closed form, the iron center of the FeGP cofactor lies in front of the *Re* face of methylene-$H_4$MPT, thereby contacting the imidazolidine ring within a distance (3 Å) reasonable for a hydride transfer. The hydride-accepting C14a atom is next to the iron coordination site, *trans* to the acyl carbon atom of the FeGP cofactor (Figure 10C), which strongly suggests this site as the binding position for $H_2$. In the crystal structures of wild-type and C176A [Fe]-hydrogenase, this iron site is occupied by a solvent molecule and a 2-hydroxyl group of DTT, respectively. If the geometric consideration of the $H_2$ binding site is correct, the unknown ligation site cannot be the location for the second intrinsic CO ligand that has also been considered until now (see Figure 2).[12] Consequently, the iron site in the wild-type holoenzyme positioned *trans* to the pyridinol nitrogen atom[10] has to be the coordination site of the second intrinsic CO ligand, as already found in the C176A-holoenzyme structure.

In the modeled closed form of the binary complex, the active site is located in a cavity that is accessible to the bulk solvent through a narrow hydrophobic channel with a length of ca. 10 Å and a diameter of ca. 3.5 Å.[12] The channel is formed by segments of the peripheral and central units, and its entrance is opposite to that of methylene-$H_4$MPT. It is perhaps used by $H_2$. Such hydrophobic channels for $H_2$ diffusion have also been found in [NiFe]- and [FeFe]-hydrogenases.[41]

## Proposed Catalytic Mechanism

A structure-based mechanism of [Fe]-hydrogenase has to integrate the following experimental results summarized by Vogt et al.:[16] (i) $H_2$ can interact with the active-site iron center only after the binding of methenyl-$H_4$MPT$^+$ to the enzyme, (ii) in the binary complex, the C14a atom of methenyl-$H_4$MPT$^+$ (and also methylene-$H_4$MPT) and the iron of the FeGP cofactor have to be juxtaposed at a suitable distance at the active site, and (iii) the proton derived from the heterolytic cleavage of $H_2$ is exchanged quickly with a proton in the bulk solvent. The structural data completely meet these conditions, and a hypothetical reaction mechanism is outlined in Figure 11.[12]

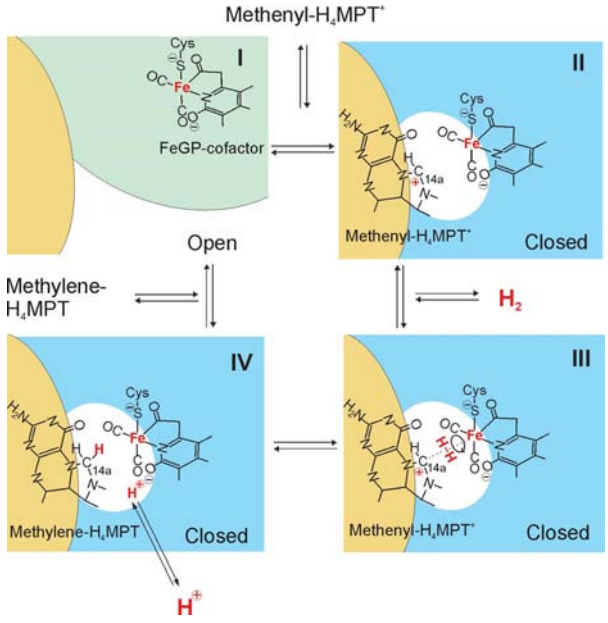


Figure 11. Proposed catalytic mechanism of [Fe]-hydrogenase involving an open/closed conformational form. (I) Before binding of the substrates, the holoenzyme is in the open form. The peripheral and central units are shown in green and ochre, respectively. (II) Upon binding of methenyl-$H_4$MPT$^+$, the active-site cleft between the two globular units is closed. (III) $H_2$ reaches the active site through the hydrophobic channel and binds to the "open coordination" site of the iron atom. A rotation of the $H_2$ molecule might explain the double $H^+$ exchange.[16] (IV) The carbocationic C14a atom of methenyl-$H_4$MPT$^+$ accepts the hydride on its *Re* face and thereby generates methylene-$H_4$MPT. The proton produced is bound to an adjacent base in the closed form and quickly exchanges with protons in bulk solvent by a proton relay pathway. Upon opening the cleft, methylene-$H_4$MPT is released to the bulk solvent. The figure was modified from that by Hiromoto et al.[12]

The catalytic cycle is initiated by the binding of methenyl-$H_4$MPT$^+$ to the open form, which triggers the closure of the cleft and thereby a change in conformation. As a result, the carbocationic character of the C14a atom increases. Subsequently, $H_2$ is supplied through a narrow hydrophobic channel to the active site in the closed form and is captured at the "open coordination" site of the iron center. The $H_2$ molecule probably binds side-on to the iron, becomes somewhat polarized, and is heterolytically cleaved by the adjacent C14a carbocation acting as a Lewis acid, as proposed by Berkessel and Thauer.[16,38,39]

The base of the reaction might be the deprotonated form of the Cys176 thiol, which is reminiscent of the terminal cysteine-thiolate ligand on the nickel atom of [NiFe]-hydrogenase that was hypothesized as proton acceptor.[42] A possible proton acceptor from the Cys176 thiol could be the neighboring Asp251 carboxylate group. Alternatively, the pyridinol hydroxylate group is an attractive candidate owing to its direct contact to the potential $H_2$-binding site and its possible interaction with the side chain of His14. An H14A exchange reduces the activity of the wild-type enzyme to less than 1%,[10] and therefore His14 may function as a proton relay from the hydroxylate group to the bulk solvent. Yang and Hall proposed a dual-pathway cleavage

  

mechanism from DFT calculations,[13] with proton transfer to Cys176 thiol and the oxygen of 2-pyridinol upon heterolytic cleavage of $H_2$ captured at the site *trans* to the acyl-carbon ligand.

## Novel Features of FeGP Cofactor Obtained from the Crystal Structures

Based on the crystal structure of [Fe]-hydrogenase, novel structural and catalytic features of the iron center have been proposed. The acyl-carbon ligand exhibits a rare biological metal–carbon σ-bonding, which should have specific structural and mechanistic functions. The catalytically relevant closed conformation of the enzyme is modeled on the basis of the known crystal structure of the apoenzyme. The spatial arrangement of the imidazolidine ring, in particular the C14 atom of the substrate and the iron complex in the closed model, suggest a possible $H_2$-ligation site. The proposed $H_2$-binding site is located *trans* to the acyl-carbon ligand. In [FeFe]- and [NiFe]-hydrogenases, one of the CO ligands is bound to the site *trans* to the $H_2$-binding site. In particular, the arrangement of the bridging CO ligand between the two iron sites in [FeFe]-hydrogenase is very similar to the acyl-carbon ligand in [Fe]-hydrogenase, which suggests common functions (Figure 12). Recent DFT calculations confirm a rather similar first ligation shell of the reacting iron center of [Fe]-hydrogenase and of [FeFe]-hydrogenase.[43]

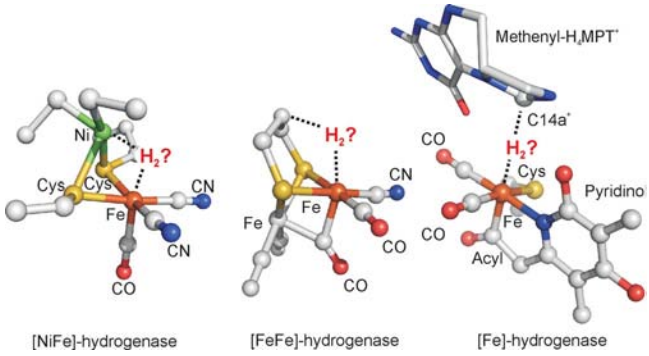


Figure 12. Structures of the active center of hydrogenases. [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* (1YRQ).[44] [FeFe]-hydrogenase from *Clostridium pasteurianum* (3C8Y).[45] [Fe]-hydrogenase from *Methanocaldococcus jannaschii* (3F47) in the closed model.[7,10,12] All three hydrogenase types have a low-spin iron atom (brown) ligated by thiolate(s), CO, and cyanide or a functional analogue of cyanide (pyridinol or acyl carbon atom) in common. The possible $H_2$-binding sites are shown, and potential interactions between $H_2$ and adjacent atoms are depicted with broken lines. Only the head part of the methenyltetrahydromethanopterin stick model (the pterin and imidazolidine rings) is shown, where the methyl groups are omitted and the C14a atom is shown as a ball.

The dinuclear arrangements of the two cations, the carbocation and the $Fe^{II}$ ion of the FeGP cofactor, in the active conformation are similar to those of the active-site dinuclear metal ions of [NiFe]- and [FeFe]-hydrogenases. Moreover, the iron ions in the three hydrogenases are ligated with CO and $CN^-$ (or functionally homologous pyridinol nitrogen and acyl carbon atoms). The similar structures of the active sites, despite the different phylogenetic origin of the enzymes, strongly suggest a convergent evolutionary development. This finding assigns a crucial function to such dinuclear-cation arrangements and the conserved low-spin iron ligation structure.

## Prospects

To confirm the closed model, crystal structures of a closed [Fe]-hydrogenase–methylene-$H_4$MPT complex at high resolution would be valuable. Conformational changes of the FeGP cofactor and the multicyclic substrate, especially the configuration of the N5 and N10 nitrogen atoms, the detailed microenvironment of the $H_2$-binding site, and the structure of the hydrophobic cavity would certainly expand our current mechanistic knowledge. Moreover, the second intrinsic CO-binding site proposed to be located *trans* to the pyridinol group has to be substantiated. A high occupancy of CO and high-resolution data are required to distinguish the solvent ligand and the CO ligand. Crystal structures of [Fe]-hydrogenase–inhibitor complexes should provide information on the ligand-exchange and dynamic properties of the Fe complex. A cyanide-inhibited enzyme structure has already been determined, but the binding site of the cyanide ion has not been reliably identified. High-resolution crystal structures of enzyme–inhibitor complexes are indispensable.

## Acknowledgments

This work was supported by the Max Planck Society, the Fonds der Chemischen Industrie, the BMBF ($BioH_2$ project), and the PRESTO Program, Japan Science and Technology Agency (JST). S. S. was financed by an emeritus grant to R. K. Thauer from the Max Planck Society; we also thank R. K. Thauer for continuous discussions and critical reading of the manuscript. We thank Hartmut Michel for continuous support.

[1] P. M. Vignais, B. Billoud, J. Meyer, *FEMS Microbiol. Rev.* **2001**, *25*, 455–501.
[2] J. C. Fontecilla-Camps, A. Volbeda, C. Cavazza, Y. Nicolet, *Chem. Rev.* **2007**, *107*, 4273–4303.
[3] S. Shima, R. K. Thauer, *Chem. Rec.* **2007**, *7*, 37–46.
[4] C. Zirngibl, W. van Dongen, B. Schwörer, R. von Bünau, M. Richter, A. Klein, R. K. Thauer, *Eur. J. Biochem.* **1992**, *208*, 511–520.
[5] R. K. Thauer, *Microbiology* **1998**, *144*, 2377–2406.
[6] Y. S. Guo, H. X. Wang, Y. M. Xiao, S. Vogt, R. K. Thauer, S. Shima, P. I. Volkers, T. B. Rauchfuss, V. Pelmenschikov, D. A. Case, E. E. Alp, W. Sturhahn, Y. Yoda, S. P. Cramer, *Inorg. Chem.* **2008**, *47*, 3969–3977.
[7] T. Hiromoto, K. Ataka, O. Pilak, S. Vogt, M. S. Stagni, W. Meyer-Klaucke, E. Warkentin, R. K. Thauer, S. Shima, U. Ermler, *FEBS Lett.* **2009**, *583*, 585–590.
[8] M. Korbas, S. Vogt, W. Meyer-Klaucke, E. Bill, E. J. Lyon, R. K. Thauer, S. Shima, *J. Biol. Chem.* **2006**, *281*, 30804–30813.
[9] E. J. Lyon, S. Shima, R. Boecher, R. K. Thauer, F. W. Grevels, E. Bill, W. Roseboom, S. P. J. Albracht, *J. Am. Chem. Soc.* **2004**, *126*, 14239–14248.

  

[10] S. Shima, O. Pilak, S. Vogt, M. Schick, M. S. Stagni, W. Meyer-Klaucke, E. Warkentin, R. K. Thauer, U. Ermler, *Science* **2008**, *321*, 572–575.
[11] C. Zirngibl, R. Hedderich, R. K. Thauer, *FEBS Lett.* **1990**, *261*, 112–116.
[12] T. Hiromoto, E. Warkentin, J. Moll, U. Ermler, S. Shima, *Angew. Chem. Int. Ed.* **2009**, *48*, 6457–6460.
[13] X. Z. Yang, M. B. Hall, *J. Am. Chem. Soc.* **2009**, *131*, 10901–10908.
[14] A. R. Klein, G. C. Hartmann, R. K. Thauer, *Eur. J. Biochem.* **1995**, *233*, 372–376.
[15] B. Schwörer, V. M. Fernandez, C. Zirngibl, R. K. Thauer, *Eur. J. Biochem.* **1993**, *212*, 255–261.
[16] S. Vogt, E. J. Lyon, S. Shima, R. K. Thauer, *J. Biol. Inorg. Chem.* **2008**, *13*, 97–106.
[17] G. C. Hartmann, E. Santamaria, V. M. Fernández, R. K. Thauer, *J. Biol. Inorg. Chem.* **1996**, *1*, 446–450.
[18] J. Schleucher, B. Schwörer, R. K. Thauer, C. Griesinger, *J. Am. Chem. Soc.* **1995**, *117*, 2941–2942.
[19] G. Buurman, S. Shima, R. K. Thauer, *FEBS Lett.* **2000**, *485*, 200–204.
[20] E. J. Lyon, S. Shima, G. Buurman, S. Chowdhuri, A. Batschauer, K. Steinbach, R. K. Thauer, *Eur. J. Biochem.* **2004**, *271*, 195–204.
[21] S. Shima, E. J. Lyon, R. K. Thauer, B. Mienert, E. Bill, *J. Am. Chem. Soc.* **2005**, *127*, 10430–10435.
[22] X. F. Wang, Z. M. Li, X. R. Zeng, Q. Y. Luo, D. J. Evans, C. J. Pickett, X. M. Liu, *Chem. Commun.* **2008**, 3555–3557.
[23] M. Salomone-Stagni, F. Stellato, C. M. Whaley, S. Vogt, S. Morante, S. Shima, T. B. Rauchfuss, W. Meyer-Klaucke, *Dalton Trans.* **2010**, *39*, 3057–3064.
[24] N. Nakatani, Y. Nakao, H. Sato, S. Sakaki, *Chem. Lett.* **2009**, *38*, 958–959.
[25] S. Shima, E. J. Lyon, M. S. Sordel-Klippert, M. Kauss, J. Kahnt, R. K. Thauer, K. Steinbach, X. L. Xie, L. Verdier, C. Griesinger, *Angew. Chem. Int. Ed.* **2004**, *43*, 2547–2551.
[26] O. Pilak, B. Mamat, S. Vogt, C. H. Hagemeier, R. K. Thauer, S. Shima, C. Vonrhein, E. Warkentin, U. Ermler, *J. Mol. Biol.* **2006**, *358*, 798–809.
[27] J. M. Rawson, R. E. P. Winpenny, *Coord. Chem. Rev.* **1995**, *139*, 313–374.
[28] B. Li, T. B. Liu, C. V. Popescu, A. Bilko, M. Y. Darensbourg, *Inorg. Chem.* **2009**, *48*, 11283–11289.
[29] T. B. Liu, B. Li, C. V. Popescu, A. Bilko, L. M. Perez, M. B. Hall, M. Y. Darensbourg, *Chem. Eur. J.* **2010**, *16*, 3083–3089.
[30] B. V. Obrist, D. F. Chen, A. Ahrens, V. Schunemann, R. Scopelliti, X. L. Hu, *Inorg. Chem.* **2009**, *48*, 3514–3516.
[31] A. M. Royer, T. B. Rauchfuss, D. L. Gray, *Organometallics* **2009**, *28*, 3618–3620.
[32] D. Chen, R. Scopelliti, X. Hu, *Angew. Chem. Int. Ed.* **2010**, *49*, 7512–7515.
[33] P. J. Turrell, J. A. Wright, J. N. T. Peck, V. S. Oganesyan, C. J. Pickett, *Angew. Chem. Int. Ed.* **2010**, *49*, 7508–7511.
[34] R. K. Thauer, A. R. Klein, G. C. Hartmann, *Chem. Rev.* **1996**, *96*, 3031–3042.
[35] S. Bartoschek, G. Buurman, R. K. Thauer, B. H. Geierstanger, J. P. Weyrauch, C. Griesinger, M. Nilges, M. C. Hutter, V. Helms, *ChemBioChem* **2001**, *2*, 530–541.
[36] P. Acharya, M. Goenrich, C. H. Hagemeier, U. Demmer, J. A. Vorholt, R. K. Thauer, U. Ermler, *J. Biol. Chem.* **2005**, *280*, 13712–13719.
[37] S. Bartoschek, G. Buurman, B. H. Geierstanger, J. Lapham, C. Griesinger, *J. Am. Chem. Soc.* **2003**, *125*, 13308–13309.
[38] A. Berkessel, *Curr. Opin. Chem. Biol.* **2001**, *5*, 486–490.
[39] A. Berkessel, R. K. Thauer, *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2247–2250.
[40] X. Z. Yang, M. B. Hall, *J. Am. Chem. Soc.* **2008**, *130*, 14036–14037.
[41] J. C. Fontecilla-Camps, P. Amara, C. Cavazza, Y. Nicolet, A. Volbeda, *Nature* **2009**, *460*, 814–822.
[42] W. Lubitz, E. Reijerse, M. van Gastel, *Chem. Rev.* **2007**, *107*, 4331–4365.
[43] M. T. Stiebritz, M. Reiher, *Inorg. Chem.* **2010**, *49*, 5818–5823.
[44] A. Volbeda, L. Martin, C. Cavazza, M. Matho, B. W. Faber, W. Roseboom, S. P. J. Albracht, E. Garcin, M. Rousset, J. C. Fontecilla-Camps, *J. Biol. Inorg. Chem.* **2005**, *10*, 239–249.
[45] A. S. Pandey, T. V. Harris, L. J. Giles, J. W. Peters, R. K. Szilagyi, *J. Am. Chem. Soc.* **2008**, *130*, 4533–4540.

Received: September 8, 2010
Published Online: December 17, 2010

  