[NiFe]- and [FeS]-cofactors in the membrane-bound hydrogenase of *Ralstonia eutropha* investigated by X-ray absorption spectroscopy: insights into O₂-tolerant H₂-cleavage

Johannes Fritsch¹, Simone Löscher², Oliver Sanganas², Elisabeth Siebert³, Ingo Zebger³, Matthias Stein⁴, Marcus Ludwig¹, Antonio L. De Lacey⁵, Holger Dau², Bärbel Friedrich¹, Oliver Lenz¹, and Michael Haumann^{2&*}

¹Humboldt-Universität zu Berlin, Institut für Biologie/Mikrobiologie, 10115 Berlin, Germany

²Freie Universität Berlin, Institut für Experimentalphysik, 14195 Berlin, Germany

³Technische Universität Berlin, Max-Volmer Institut, 10623 Berlin, Germany

⁴Max-Planck-Institut für Dynamik komplexer technischer Systeme, 39106 Magdeburg, Germany

⁵Instituto de Catálisis y Petroleoquímica (CSIC), 28049 Madrid, Spain

[&]Financial support by the Deutsche Forschungsgemeinschaft (DFG) (SFB498-C1, -C8, -C9; Ha3265/3-1 to M.H.; Unicat Cluster of Excellence Berlin), the EU/Energy Network SolarH2, and the Bundesministerium für Bildung und Forschung (Design Cells Consortium) is gratefully acknowledged. M.H. thanks the DFG for a Heisenberg Fellowship. A.L.L. thanks the Spanish MICIIN for financial support (project CTQ2009-12649). M.S. is grateful to the Max Planck Society for the Advancement of Science for financial support.

**Correspondence to:*

Dr. Michael Haumann, Freie Universität Berlin, FB Physik, Arnimallee 14, 14195 Berlin, Germany,
Phone: +49 30 838 56101, Fax: +49 30 838 56299, Email: michael.haumann@fu-berlin.de

Keywords: hydrogenase, O₂-tolerance, [NiFe] active site, FeS cluster, X-ray absorption spectroscopy

Running Title: Insights from XAS into O₂-tolerance of [NiFe] hydrogenase

Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; DFT, density functional theory; PH, periplasmic [NiFe] H₂ase of *D. gigas*; FTIR, Fourier-transform infrared spectroscopy; H₂ase, hydrogenase; MBH, membrane-bound [NiFe] H₂ase of *R. eutropha*; TXRF, total-reflection X-ray fluorescence detection; XANES, X-ray absorption near-edge structure; XAS, X-ray absorption spectroscopy.

Abstract:

Molecular features that enable certain [NiFe] hydrogenases to catalyze hydrogen (H₂) conversion in the presence of dioxygen (O₂) were investigated in the present study. By means of X-ray absorption spectroscopy (XAS) we compared the [NiFe] active site and FeS clusters in the O₂-tolerant membrane-bound hydrogenase (MBH) of *Ralstonia eutropha* and the O₂-sensitive periplasmic hydrogenase (PH) of *Desulfovibrio gigas*. Fe-XAS indicated an unusual complement of iron-sulfur centers in the MBH, likely based on an unusual structure of the FeS cluster proximal to the active site. This cluster is a [4Fe4S] cubane in PH. For MBH it comprises less ~2.7 Å Fe-Fe distances and additional longer vectors of ≥3.4 Å, consistent with an Fe-trimer plus a more isolated Fe ion. Ni-XAS indicated a similar architecture of the [NiFe] site in MBH and PH, featuring Ni-coordination by four thiolates of conserved cysteines, i.e. in the fully reduced state (Ni-SR). For oxidized states, short Ni-μO bonds due to Ni-Fe bridging oxygen species were detected in the Ni-B state of the MBH and in Ni-A of the PH. Furthermore, a bridging sulfenate (CysSO) is suggested for an inactive state (Ni_{ia}-S) of the MBH. We propose that the O₂-tolerance of the MBH is mainly based on a dedicated electron donation from a modified proximal FeS cluster to the active site, which may favour formation of the rapidly re-activated Ni-B state instead of slowly re-activated Ni-A. Thereby, catalytic activity of the MBH is facilitated in the presence of both, H₂ and O₂.

Biochemistry 50: 5858- 5869 (2011)

Hydrogenases (H_2 ases) are enzymes that catalyze the cleavage and production of molecular hydrogen (H_2) at high turnover rates (1-2). They are of interest in the worldwide effort to substitute fossil fuels by renewable energy sources, such as H_2 (3-5). A limitation for biotechnological application of most H_2 ases is their sensitivity towards dioxygen (O_2) (6). However, a few H_2 ases are catalytically active in the presence of O_2 (7-11). The elucidation of the molecular basis of their O_2 -tolerance may open new strategies to improve the catalytic features of O_2 -sensitive H_2 ases by genetic engineering (12-15) and could be useful for the design of novel biomimetic synthetic catalysts (16-18).

The facultative chemolithoautotrophic bacterium *Ralstonia eutropha* H16 (*Re*) has the ability to grow on H_2 , CO_2 , and O_2 , and harbours three H_2 ases, all of which are active in the presence of O_2 . These are the regulatory H_2 ase (RH), which acts as an H_2 sensor (19-21), the soluble NAD^+ -reducing H_2 ase (SH) (22-24), and the membrane-bound H_2 ase (MBH) (25-26). All enzymes belong to the [NiFe] class and their active sites contain one Ni and one Fe atom (1, 27-29) as opposed to the [FeFe] H_2 ases possessing a homo-metallic center (1, 27, 30). The MBH, which is in the focus of the present study, is bound to the periplasmic side of the cytoplasmic membrane via a membrane-integral *b*-type cytochrome (31). The enzyme shows high H_2 -oxidation activity (26) and feeds the derived electrons into the respiratory chain (32).

The MBH sustains growth of *R. eutropha* with H_2 even at atmospheric levels of O_2 (26, 33-35). Electrochemical experiments have shown that H_2 -oxidizing activity at ambient O_2 pressure (~230 mbar) can be up to 60 % of the activity determined in the absence of O_2 ; nearly full activity is recovered at high redox potential when O_2 is replaced by H_2 (33-36). Furthermore, the MBH is insensitive to inhibition by CO (36). These features are unique and not found for the standard type of [NiFe] H_2 ases, e.g., from *Desulfovibrio* and *Allochromatium* species, in which H_2 -oxidizing activity is completely inhibited by traces of O_2 and low CO levels. Reactivation of O_2 -inhibited standard H_2 ases occurs extremely slowly and only at low potentials (6, 37-38).

Comentario [MSOffice1]: ?

The high O₂-tolerance of the MBH is surprising because its amino acid sequence (26, 29) suggests a high structural similarity with standard [NiFe] H₂ases, e.g. the periplasmic H₂ase (PH) of *Desulfovibrio gigas* (Fig. 1). The MBH consists of a large subunit (HoxG) comprising the [NiFe] site and a small subunit (HoxK), which harbours binding sites for three FeS clusters (Fig. 1) (39-40). According to the crystal structures of several [NiFe]-hydrogenases, generally four conserved cysteines in the large subunit are involved in coordinating the active site Ni and Fe atoms (27, 41). A further common feature of [NiFe] H₂ases is the ligation of the Fe by two cyanides (CN⁻) and one CO as observed by FTIR spectroscopy (36, 42-43). Apart from these similarities, biochemical, electrochemical, and spectroscopic results (26, 36, 43-45) suggest that O₂ tolerance of the MBH is related to the structural arrangement of its metal cofactors, rather than to the restricted access of O₂ and CO to the [NiFe] site due to a narrow gas-channel as proposed for H₂-sensing H₂ases (12, 26, 46). Notably, compared to standard H₂ases, biosynthesis of active MBH requires a significantly larger set of maturation proteins (47-49).

Spectroscopic studies on the MBH using EPR and FTIR techniques uncovered several features differing from those of standard enzymes (43-46). The so-called Ni-A state, corresponding to oxidized, inactive unready enzyme, was not detectable in wild-type MBH. For standard H₂ases residing in the Ni-A state, a (hydro)peroxo species was suggested to occupy the bridging position between Ni and Fe, whereas in the Ni-B state a hydroxide is assigned as bridging ligand (50). Remarkably, in addition to the absence of Ni-A, a complex EPR spectrum indicates modification of the FeS cluster in the proximal position to the [NiFe] site in the MBH (43-46). The structural basis of these modifications needs to be elucidated.

In the present study, the metal centers of the MBH were characterized by an element-specific technique, namely X-ray absorption spectroscopy (XAS) (51-52), by which the coordination environment and redox changes of the protein-bound metal atoms were determined. The spectroscopic features of the MBH are compared to those of the PH standard

Biochemistry 50: 5858- 5869 (2011)

H₂ase, for which crystal structures are available (27, 53). Our results suggest a different structure of the proximal FeS cluster, but a rather similar, although more disordered [NiFe] active site in MBH compared to PH.

MATERIALS AND METHODS

Enzyme purification and sample preparation. Three different MBH protein preparations were used for this study. The most active MBH (mean activity of 140±13 U/mg) was isolated from *R. eutropha* strain HF649, which was grown on fructose-glycerol mineral medium under oxygen-limited conditions (43-44, 54). The membrane fraction was prepared under an argon atmosphere and oxidized by the addition of 50 mM K₃[Fe(CN)₆] prior to aerobic solubilisation with Triton X-114. Purification via *Strep*-Tactin affinity chromatography was carried out in K-PO₄ buffer, pH 7.0. The protein samples were concentrated using Amicon Ultra-15 (PL-30) and Amicon Microcon (YM-30) filtration devices (Millipore) in buffer containing 40 mM K-PO₄, pH 5.5, 150 mM NaCl, 20 % glycerol. MBH mutant protein (HoxK Cys19/120Gly) was prepared as previously described (54) and concentrated as shown above (pH 5.5, ~86 U/mg). Less active MBH protein (mean activity of 60±4 U/mg) was purified as described previously (26, 43) and concentrated under aerobic conditions, and the purified protein was finally stored in 50 mM K-PO₄ buffer at pH 8.0. All preparations lacked the membrane-integral cytochrome-*b* that serves as primary electron acceptor. Protein concentrations were determined by the Bradford method (55) with bovine serum albumin as a standard. Purity of samples was examined by SDS-PAGE. H₂ase activity was determined photometrically (26, 40) in 50 mM K-PO₄ buffer at pH 5.5 using the artificial electron acceptor methylene blue. For treatments in the presence of H₂, concentrated protein solutions (~30 µL) were enclosed in gas-tight reaction vessels, the atmosphere was exchanged by repeated degassing (23), and solutions were incubated under a gentle stream of moistured gas for 10-45 min at ~20 °C. The final MBH protein concentrations in the samples

Biochemistry 50: 5858- 5869 (2011)

were 0.5-0.8 mM. PH protein from *D. gigas* was prepared and concentrated under aerobic conditions and protein concentrations were determined as described before (56). Activation of PH was performed upon incubating Eppendorf reaction vessels containing concentrated protein (~30 μ l) in repeatedly degassed buffer at 35 °C under pure H₂ for 90-180 min. Reduction of PH was achieved by incubation with Na-dithionite (10 mM) for 20 min. Final PH concentrations were 0.7-1.0 mM. Immediately after the treatments, ~20 μ l of protein samples were transferred under argon atmosphere to Kapton-covered sample holders for XAS and EPR measurements, rapidly frozen, and stored in liquid nitrogen until use.

Metal content quantification. The metal content in H₂ase proteins was determined by total-reflection X-ray fluorescence detection (TXRF) (57) on a PicoFox instrument (Bruker). Ni and Fe concentrations were determined using the built-in spectrometer functions relative to a gallium standard (Sigma), which had been added prior to the measurements to the protein solutions.

FTIR spectroscopy. Infrared spectroscopy was carried out on a Bruker TENSOR 27 spectrometer as described before (43) with aliquots of the protein samples used for XAS.

X-ray absorption spectroscopy (XAS). XAS measurements were performed at beamline D2 of the EMBL outstation (at HASYLAB, DESY, Hamburg, Germany) and at beamline KMC-1 of BESSY (Helmholtz-Zentrum Berlin). *K α* fluorescence-detected XAS spectra at the Ni and Fe K-edges were collected for samples held in liquid-helium cryostats at 20 K with energy-resolving 13-element Ge detectors (Canberra) (58-59). Fe spectra were recorded for a monochromator scan range of 7000-8200 eV. For Ni, in an extended-range approach (60), the scan range was 8200-9200 eV. XAS spectra were averaged (5-12 scans) after energy calibration of each scan by the Bragg-reflection method (61) or using the spectrum of a Ni metal foil as an energy standard, normalized, and EXAFS oscillations were extracted (52). The energy scale of EXAFS spectra was converted to a wavevector (*k*) scale using *E*₀ values of 8333 eV (Ni) and 7112 eV (Fe) *E*₀ refined to ~7120 eV (Fe) and ~8336 eV (Ni) in the

simulations. Unfiltered k^3 -weighted spectra were used for least-squares curve-fitting and Fourier-transform (FT) calculation with the in-house program SimX (52). In EXAFS simulations, phase-functions from FEFF7 (62) and amplitude reduction factors (S_0^2) of 0.9 (Ni) and 0.85 (Fe) were used.

XANES simulations. XANES calculations were done as described in (23, 63) using the code FEFF8.2 (62) with the full-multiple-scattering (FMS) and the self-consistent-field (SCF) options activated. Atomic coordinates of FEFF input files were generated using the crystal structure of the [NiFe] site of *D. gigas* H₂ase (41) as a template and Ni-ligand distances from the EXAFS analysis. Calculated spectra were shifted by 1 eV to lower energies and smoothed over data points within 3 eV for better comparison with the experimental data.

Density functional theory (DFT) calculations. DFT calculations were performed using the program ORCA (64-66). Geometry optimizations of [NiFe] site models involved the BP86 exchange-correlation functional (67-68) and a double-zeta basis set (69) with polarization functions from the TURBOMOLE library (<ftp://ftp.chemie.uni-karlsruhe.de/pub/basen>) and a dielectric constant of $\epsilon = 4$ in a COSMO solvation model (70, 71).

RESULTS

The FeS clusters studied by XAS at the iron K-edge. By Fe-XAS we compared the properties of FeS clusters in the *R. eutropha* MBH with those of the *D. gigas* PH (Fig. 2). In the XANES spectrum of oxidized MBH, the primary maximum at ~7129 eV was larger, the edge energy at 50 % level was by ~0.6 eV higher (7118.2 eV), and the energy of the pre-edge feature due to $1s \rightarrow 3d$ electronic transitions was by ~0.4 eV higher (7112.1 eV), compared to the respective parameters of the PH spectrum (Fig. 2C). These differences suggest alterations in the average iron coordination and an overall higher content of oxidized Fe ions in the MBH. In H₂-reduced MBH, the edge and pre-edge feature were shifted by ~0.9 eV and ~0.6

eV to lower energies (7117.3 eV and 7111.5 eV), indicating significant Fe reduction in this sample (Fig. 2C).

By TXRF, an Fe per Ni ratio of 12.1 ± 0.3 was determined for the PH, which is in agreement with the expected 12 Fe ions (deduced from two [4Fe4S] and one [3Fe4S] clusters and the active site) per Ni. For the MBH, a slightly lower ratio of 11.1 ± 0.8 was determined. However, even a small Ni contamination of 5 % of the protein-bound metal would diminish the ratio by ~ 0.5 units and result in an underestimation of the metal ratio. Thus, a conservative estimate is a content of 12 Fe ions for the MBH, similar to the PH. Taking into account this value and an expected edge shift of about 3 eV for a single-electron redox change of a mono-iron compound (72), the data are compatible with the presence of one to two higher oxidized ions, i.e. Fe(III), in the oxidized MBH compared to the PH and with the reduction of about three iron ions from Fe(III) to Fe(II) in the case of the MBH upon H₂-incubation.

EXAFS analysis was employed to study the structure of the Fe sites (Fig. 2). The Fourier-transforms (FTs) of the respective EXAFS spectra of oxidized MBH and PH were dominated by FeS cluster contributions, exhibiting two distinct FT peaks related to Fe-S (I) and Fe-Fe (II) distances (21, 58). For the oxidized MBH, both FT-peak amplitudes were largely diminished, suggesting an overall structure of the FeS clusters different from that in the PH. For H₂-reduced MBH, an increase of FT peak I was observed. However peak II again was much smaller than for the PH (Fig. 2A). Fe-Fe and Fe-ligand distances (R_i) and their numbers per Fe atom (coordination number N_i) were determined by curve-fitting of the EXAFS oscillations (48). The spectrum of the PH was in full agreement with the corresponding simulations (Table 1-I,II) predicting two [4Fe4S] and one [3Fe4S] clusters in this enzyme. In particular, the coordination number ($N_{\text{Fe-Fe}}$) of Fe-Fe distances of ~ 2.7 Å, typical for the above mentioned FeS clusters, closely matched the value of ~ 2.6 , which was calculated for the PH (see below). In addition, the inter-atomic distances were similar to those in crystal structures (53) and previous XAS data (73).

Simulation of the EXAFS spectrum of oxidized MBH using the same FeS complement as for the PH was insufficient (2-fold increased error, R_F ; Table 1-III). A better fit quality was obtained using the Debye-Waller parameters ($2\sigma^2$) of the PH as a reference, but varying the individual N_i -values (Table 1-IV). A coordination number $N_{\text{Fe-Fe}}$ of ~ 1.6 was obtained for the MBH, which is by approximately one unit lower than in the PH. A similar fit (not shown) was obtained for the less active MBH preparation at pH 8.0 (Fig. 2A). Accordingly, major differences in the average iron coordination spheres in the two MBH preparations were not observed by XAS. The $N_{\text{Fe-Fe}}$ value of the MBH appeared to be too low to accommodate two [4Fe4S] and one [3Fe4S] clusters, suggesting an apparent diminishing of the Fe-Fe contributions to the MBH EXAFS by interference effects, e.g., of Fe-S and Fe-Fe interactions.

For the first-sphere iron coordination in MBH, an apparent number of Fe-S bonds was obtained that was also too low to account for the four sulfurs ligating the Fe ions in conventional FeS clusters (Table 1-III,IV). However, the number of iron interactions with light scatterers due to the Fe-C(=O/N)/O bonds at the active-site iron was similar for MBH and PH, and thus there was no evidence for oxidatively modified FeS clusters in the MBH. A more elaborated EXAFS simulation approach included two Fe-S and Fe-Fe shells. This approach revealed additional longer iron-sulfur bonds of ~ 2.6 Å and thus a total $N_{\text{Fe-S}}$ closer to the expected value of ~ 4 , and an improved fit quality. Furthermore, also the $N_{\text{Fe-Fe}}$ value was increased to close to 2 (Table 1-V), which, however, still was significantly lower than for the PH. Two Fe-Fe distances of 2.68 Å and 2.78 Å (with coordination numbers close to unity) and a longer distance of ~ 3.4 Å with smaller coordination number now became discernable (Table 1-V,VI). The Debye-Waller parameter (σ) (Table 1) suggested a distance distribution of ± 0.06 Å for the Fe-Fe interactions. Thus, particularly short Fe-Fe distances of ~ 2.6 Å and longer distances of ~ 2.85 Å presumably are present in the MBH, both of which were not detectable in the PH. However, the conventional Fe-Fe distance of ~ 2.7 Å was observed in both enzymes.

Simulations of the EXAFS of H₂-reduced MBH using the same approach yielded a similar $N_{\text{Fe-Fe}}$ close to 2 as for oxidized enzyme (Table 1-VII,VIII). The increase in FT peak I was explained by homogenization of the Fe-S bond lengths, i.e. a lower number of longer Fe-S bonds, in agreement with the reduction of several Fe ions observed in the XANES.

Figure 2 shows also the EXAFS spectrum of an MBH variant, in which two cysteines (Cys19 and Cys120) were replaced by glycines in the small subunit HoxK by genetic engineering (54). These particular cysteines are absent in the PH and appear to be located close to the proximal FeS cluster in the MBH. For the respective mutant protein, MBH^{C19G/C120G}, a significantly increased oxygen-sensitivity has been reported previously (54). The Fe/Ni ratio of MBH^{C19G/C120G} preparations was 11.6 ± 0.3 , which is close to the 12 Fe ions expected according to the *D. gigas* structure. The FT spectrum of the mutant protein in particular revealed an increased peak II, more similar to the PH. Accordingly, the EXAFS simulation yielded a larger $N_{\text{Fe-Fe}}$ than in wildtype MBH. In the mutant protein the Fe-Fe distances did not exceed ~ 3.0 Å (Table 1-IX). In summary, the overall structure of FeS clusters in this MBH variant was significantly different from that in wildtype MBH and more similar to that in the PH.

For the PH, a total number of 31 Fe-Fe/Ni distances with a length of about ~ 2.7 Å was calculated (each Fe-Fe pair contributes two distances; 2×12 plus 1×6 distances in the two [4Fe4S] and one [3Fe4S] clusters and the Ni-Fe distance with a similar length and therefore indistinguishable from the Fe-Fe interactions; see next section). For the MBH, a comparable calculation based on 12 Fe ions and on the data displayed in Table 1 yielded ~ 25 Fe-Fe/Ni distances of ~ 2.7 Å plus around four longer vectors of ~ 3.4 Å. According to EPR results, the MBH contains one [4Fe4S], one [3Fe4S] cluster, and an additional FeS cluster of unknown structure (43, 45, 54). Subtraction of the 18 Fe-Fe vectors of the [4Fe4S] and [3Fe4S] clusters and the Ni-Fe distance resulted in six Fe-Fe vectors of ~ 2.7 Å in addition to the longer interactions, which are likely attributed to the third FeS cluster. These values are incompatible

Biochemistry 50: 5858- 5869 (2011)

with an assignment of this cluster to a conventional [4Fe4S] cubane in the MBH and suggest a more unusual FeS species, most probably in the proximal position (43, 45, 54).

Ni XAS on the active site structure. Prior to XAS analysis, the population of the various states of the [NiFe] site in the samples was determined by EPR and FTIR spectroscopic techniques (data not shown, see (42-43, 45, 54, 74-75)). EPR revealed that the oxidized PH was to more than 85 % in the Ni^{III}-A state, previously interpreted as carrying a bridging peroxide species between the Ni and Fe ions (50, 76). After 90 min of reductive incubation with H₂, the Ni^{III}-C state with a hydride, H⁻ in the bridging position (77-78) was present in ~65 % of the protein; minor populations of other states summed up to ~35 %. In Na-dithionite reduced PH, most of the [NiFe] site was fully reduced (75, 79) and the respective Ni^{II}-SR states (see below) may harbor a metal-bound hydride as well (64, 80).

For the MBH, various states of the [NiFe] site have been previously assigned by FTIR spectroscopy, according to the characteristic infrared stretching frequencies of the Ni-bound CO ($\nu(\text{CO})$) (43, 45, 54, 81). Analysis of the $\nu(\text{CO})$ values of the anaerobically oxidized MBH samples at pH 5.5 revealed that the majority of the [NiFe] sites (~70 %) resided in the Ni^{III}-B state, while the remaining fraction contained Ni(II)-states (Table 2). The Ni-A state was not detected in any of the studied MBH samples, which is consistent with previous observations (43, 45, 54). In H₂-reduced MBH, the Ni-SR states were dominant (~80 %). The Ni_{ia}-S inactive state with a Ni(II) (43, 54) was the main species (~70 %) observed in aerobically purified MBH at pH 8.0. An increased Ni_{ia}-S population was also found in a sample of highly active, H₂-reduced MBH that was re-oxidized by exposure to air after XAS data collection and subsequent thawing (Table 2).

The coordination environment and oxidation state of the Ni atom as a crucial constituent of the active site was studied by XAS at the Ni K-edge. XANES spectra of the PH in the Ni-A and the MBH in the Ni-B state revealed a similar edge energy, in agreement with

the presence of Ni(III) in both species (Fig. 3). The edge shapes were typical for a dominant sulfur-coordination of the Ni (82), i.e. by the thiolates of four cysteines residues. The two enzymes showed only minor differences, suggesting a quite similar first-sphere Ni-coordination. A PH sample with a preferential Ni-C content exhibited a decreased secondary edge maximum at ~8360 eV, likely due to the replacement of the Ni-Fe bridging oxygen species by a hydride (82-84). Interestingly, the slightly higher edge energy implied an even more oxidized Ni ion in the Ni-C state compared to Ni-A. Decreased secondary edge maxima also were observed for MBH and PH in the Ni-SR state(s). Due to near-quantitative Ni(II) formation, the corresponding edge energies were around 0.5 eV lower than in the oxidized samples (Fig. 3). In the oxidized MBH at pH 8.0, a pronounced increase of the primary edge maximum at ~8350 eV suggested additional terminal oxygen ligands at the Ni(II) (23, 58, 82, 85) in the Ni_{IIa}-S state and therefore a substantially different site structure compared to Ni-B.

Applying EXAFS analysis, the bond lengths and numbers of ligands at the Ni ion were determined (Fig. 4). The Fourier-transform (FT) of the EXAFS spectrum (Fig. 4A) of the PH in the Ni-A state revealed two main peaks due to Ni-oxygen and Ni-sulfur bonds. Features at higher distances reflected contributions from the Ni-Fe vector (23, 60). The best-fit parameters (Table 3-I) showed that the Ni-A spectrum was completely described by close to one short Ni-oxygen distance, four Ni-sulfur bonds from the cysteines at the active site, and a Ni-Fe distance of 2.75 Å, all of which are similar to those found in crystal structures (50, 53). The short bond length of 1.85 Å suggested that the O-atom was in a Ni-Fe bridging position (83). Further O-atoms in the first coordination sphere of Ni were not detected. This rather excludes the presence of sulfenates (oxidized cysteines, CysSO) bound via their O-atom to Ni, which have been found in several crystal structures of oxidized *Desulfovibrio* H₂ases (86-88). Also the second O-atom of a (hydro)peroxide ligand, i.e. at ~2.2 Å to Ni, could not be observed. In the Ni-C sample of the PH (Fig. 4A, Table 3-II), the Ni-oxygen bond was completely lost (vanishing $N_{\text{Ni-O}}$). The expected Ni-hydride bond (77-78) in Ni-C can not be

detected by EXAFS because of the very small backscattering amplitude of hydrogen. A similar overall coordination of the Ni(II) was also determined for the Ni-SR states of the PH (Table 3-III). The shortening of the Ni-Fe distance by ~ 0.2 Å in Ni-C and Ni-SR was in agreement with crystal structures of reduced *Desulfovibrio* H₂ases (86, 88). In the Ni-SR state, the longest Ni-sulfur distance, which likely belongs to the axial sulfur ligand at Ni (41), was even longer than the Ni-Fe distance, as previously observed (14).

The FT of the EXAFS spectrum of the MBH in the Ni-B state was different from that of the PH in the Ni-A conformation. The considerably smaller overall amplitudes (Fig. 4B) suggested more variable Ni-sulfur bond lengths in the MBH. A corresponding simulation revealed a coordination number of the Ni-oxygen interactions of ~ 0.7 (Table 3-IV), in agreement with a Ni-B content of about 70 % in the respective sample. The main differences between the Ni-B state of the MBH and the Ni-A state of the PH were (i) a ~ 0.03 Å longer Ni-oxygen distance, (ii) a ~ 0.04 Å longer mean Ni-sulfur distance, (iii) a doubled Debye-Waller parameter ($2\sigma^2$) of the shorter Ni-sulfur interactions, which implied a larger Ni-sulfur distance spread between 2.12-2.28 Å, and (iv) a ~ 0.1 Å shorter Ni-Fe distance in the MBH of ~ 2.65 Å (Table 3-IV).

The EXAFS spectrum of H₂-reduced MBH was very similar to that of Na-dithionite-reduced PH (Fig. 4). The respective simulations revealed a similar Ni coordination in both samples and, in particular, the apparent absence of Ni-bound oxygen. The Ni-Fe distance was shortened by ~ 0.15 Å (Table 3-V) and thereby became better discernable in the FT spectra (Fig. 4B). The largely decreased $2\sigma^2$ value of the Ni-sulfur interactions indicated a homogenization of the corresponding bond lengths in the MBH in the Ni-SR state. However, the overall Ni-sulfur and Ni-Fe distances remained slightly longer than in the PH.

The FT of the EXAFS spectrum of the MBH preparation preferentially in the in Ni_{ia}-S state (pH 8.0) revealed largely diminished amplitudes, a shift of the main peak to larger distances, and a smaller peak feature due to short Ni-oxygen bonds compared to Ni-B (Fig.

4B). The simulation yielded an increased number of Ni-oxygen bonds with a longer interatomic distance of $\sim 2 \text{ \AA}$, a by $\sim 0.1 \text{ \AA}$ larger mean Ni-sulfur bond length, an increase in the coordination number of long Ni-sulfur distances to about two, and a by $\sim 0.3 \text{ \AA}$ longer Ni-Fe distance of $\sim 3.05 \text{ \AA}$, compared to the Ni-B state of the MBH (Table 3-VI). The longer oxygen bonds most likely belong to terminal Ni ligands and not to metal-bridging μO species. Two long Ni-sulfur interactions may be attributable to the axial Ni-SCys bond and to a Ni-O(SCys) binding motif (see below).

The long Ni-Fe distance and additional long Ni-sulfur distances of the MBH in $\text{Ni}_{\text{ia}}\text{-S}$ resembled the situation in crystal structures of standard [NiFe] H_2 ases showing a Ni-Fe bridging sulfenate (CysSO) (SI-1). XANES simulations on the basis of Ni-coordinations involving 0, 1, or 2 Ni-bound oxygen atoms (SI-2) readily reproduced the shape changes between Ni-SR and Ni-B in the experimental spectra of the MBH due to the loss of the bridging hydroxyl species and suggested that the increased maximum in the XANES of $\text{Ni}_{\text{ia}}\text{-S}$ reflects the presence of two non- μO oxygen ligands. By density functional theory calculations (DFT), geometry-optimized structures (not shown, see (64-65)) were obtained for the Ni-B and Ni-S states with a standard $(\text{CysS})_4$ Ni-ligation and with terminal or Ni-Fe bridging sulfenate groups (SI-3, Table S1). The resulting structures suggest that terminal sulfenates bind to Ni via their S-atom, but equatorial bridging sulfenates bind to Ni via their O-atom, in agreement with crystal data (SI-1). A bridging CysSO elongated the Ni-Fe distance to a value of $\sim 3 \text{ \AA}$ and created an additional long Ni-sulfur distance. Similar changes were observed by XAS for the $\text{Ni}_{\text{ia}}\text{-S}$ state of the MBH. Minor changes in the Ni-Fe distance were observed for a terminal CysSO compared to a standard active site. These results favor a metal-bridging CysSO group in the $\text{Ni}_{\text{ia}}\text{-S}$ state, but, however, a [NiFe] site structure without obvious cysteine modifications in the Ni-B state of the MBH.

DISCUSSION

Structure of FeS clusters in the MBH. The Fe-XAS analysis suggests different FeS cluster compositions in MBH and PH. As a main result a diminished number of Fe-Fe distances of ~ 2.7 Å, typical for conventional [4Fe4S] and [3Fe4S] clusters (21, 90), was observed in the MBH. The small subunit of the PH contains two [4Fe4S] clusters in the proximal and distal positions relative to the active site and the medial [3Fe4S] cluster, respectively (53). EPR spectroscopy revealed that the HoxK subunit of the MBH contains one [4Fe4S] and one [3Fe4S] cluster, in addition to an unusual paramagnetic iron-sulfur species. The latter interacts magnetically both with the Ni(III) of the active site in the Ni-B state and the oxidized [3Fe4S]⁺ cluster in the medial position (43, 45, 54). Accordingly, this species most likely is located at the position of the proximal cluster. Similar EPR results were obtained for other O₂-tolerant [NiFe] H₂ases (9, 11, 91-92). Site-directed mutagenesis studies on the MBH, combined with spectroscopic and electrochemical investigations consistently point to a modified proximal FeS center, which has been shown to be crucially related to O₂ tolerance (46, 54). We therefore discuss possible structural motifs for this unusual cofactor, derived from the Fe-XAS analysis.

As emphasized previously, a common feature of the small subunits of O₂-tolerant membrane-bound [NiFe] H₂ases is the presence of two additional cysteine residues, Cys19 and Cys120 in the case of the MBH from *R. eutropha*, in close vicinity to the position of the proximal FeS cluster. These particular cysteine residues are replaced by glycines in standard H₂ases (see (54) and refs. therein). However, the four conserved cysteines, ligating the proximal cluster are present both in standard [NiFe] H₂ases, such as the PH, and in the O₂-tolerant enzymes. Thus, the presence of two additional cysteine residues in coordination distance may provide a surplus of iron ligands in MBH-like O₂-tolerant H₂ases.

Fe-XAS revealed at most 6 Fe-Fe distances of 2.6-2.85 Å and up to 4 longer distances of ~ 3.4 Å for the proximal cluster of the MBH. This is incompatible with a cubane [4Fe4S]

species, for which 12 Fe-Fe distances of ~ 2.7 Å would be expected and were actually found in the PH. We note that the error in the numbers of Fe-Fe distances in the MBH may be in the order of about ± 2 , due to statistical coupling between coordination number and Debye-Waller parameter in EXAFS simulations (52). The aforementioned numbers of Fe-Fe interactions in the MBH suggest three Fe-Fe pairs with a separation of metal ions by 2.6-2.85 Å and one to two additional pairs with a separation of ~ 3.4 Å. Fe-Fe distances larger than 4 Å cannot be excluded, but are difficult to detect by XAS. Notably, there was no evidence from XAS for additional non-sulfur iron ligands in the MBH.

Based on these considerations, a similar ligation pattern of the conserved cysteines, assuming four proximal Fe ions in the MBH, and molecular modelling results of the structure of the HoxK subunit (54), a possible model for the proximal FeS cluster is suggested (Fig. 5A). It includes an Fe-trimer combined with a more remote Fe ion. The shortest detected Fe-Fe distance of ~ 2.6 Å suggests that one of the additional cysteines may provide a bridging ligand (Fe- μ S(Cys)-Fe motif), as observed for the active site H-cluster of [FeFe] H₂ases (27, 30). However, it remains unclear if four μ_3 S bonds are implemented in the structure and how the iron at about 3.4 Å distance to one or two of the other Fe ions is connected to the trimer. The loss of one μ S bridge may open a coordination site at the more distant Fe ion for the binding of an additional cysteine. Notably, such a structure (Fig. 5A) is conservative in a way that it comprises only iron ligated by sulfur ligands and requires relatively small deviations (<1 Å) of the Fe ions from the crystallographic positions in the PH (53).

Modified FeS clusters were also observed in crystal structures of standard H₂ases, but mainly attributed to protein damage due to crystallization and/or X-ray induced modifications during data collection (93-94). The presence of a special cluster also in the most native, membrane-containing preparation (43), the absence of X-ray induced damage under our XAS conditions, and similar Fe-XAS data for various protein samples argue against unspecific FeS cluster modifications in the MBH. Furthermore, the MBH variant lacking the additional

cysteines (54) reveals an iron coordination pattern more similar, although not fully identical to that of the PH. Therefore, the data suggest a more cubane-like structure of the proximal cluster. This mutant enzyme exhibited a higher sensitivity to O₂-inhibition (54), which correlates the presence of the two cysteines directly with the O₂-tolerance of the MBH (46, 54). Therefore, we propose that a structurally modified proximal FeS cluster is a native and functionally important feature of the MBH.

Properties of the [NiFe] active site. A central result of the present Ni-XAS study is that the overall structural features of the functional states, Ni^{III}-B and Ni^{II}-SR, of the [NiFe] site in the MBH are similar to those in the PH (Fig. 5B). Both revealed four sulfur ligands at Ni, likely stemming from the four conserved cysteines at the active site in both enzymes. For the Ni-B state of the MBH, a particularly short Ni-oxygen bond (Ni-μO motif) was detected, which we assign to a Ni-Fe bridging OH⁻ as proposed for standard H₂ases (50, 76). A similar conclusion has been obtained in previous EPR and FTIR studies of the MBH (43-45). Thus, the O₂-tolerance of the MBH is not based on obvious modifications of its [NiFe] site.

There are, however, more subtle structural differences. The mean Ni-sulfur bond length in the Ni-B state of the MBH was overall larger and a larger bond lengths distribution was observed, compared to the Ni-SR state of the MBH and to the Ni-A and Ni-C states of the PH. The Ni coordination in Ni-B thus is considerably less homogeneous, possibly reflecting differences in the amino acid environment of the [NiFe] site. Indeed, at position 71 in the large subunit, a valine is found in the PH, but a cysteine (C81) in the MBH (44). The V71 side chain is at ~4 Å distance to the Ni-Fe bridging C68 in the PH (50, 53). C81 could be at a similar distance to the bridging C78 in the MBH. In line with this rationale, mutations of C81 in the MBH negatively affected the affinity for the substrate H₂, but the O₂-tolerance remained unchanged (26). A somewhat increased O₂-tolerance and decreased affinity for carbon monoxide (CO), a common inhibitor of standard H₂ases but not of the MBH (26), was found for a cysteine mutant of *D. fructosovorans* (13). Weak CO binding at Ni was also

reported for the O₂-tolerant H₂ase of *A. aeolicus* (95). These results suggest that an additional cysteine close to the [NiFe] site is not sufficient to mediate full O₂-tolerance of the MBH. Rather, a fine-tuned [NiFe] site coordination may decrease the binding strength of inhibitory ligands at Ni. The longer Ni-μO bond and the smaller Ni-Fe distance suggest that also the bridging oxygen species in Ni-B of the MBH is less firmly bound than that in Ni-A of the PH. This interpretation would be even more interesting, if the Ni-A state does not comprise a bridging peroxidic species, but rather a hydroxide (see below). Thus, a direct comparison of the Ni-B state of standard H₂ases with that of MBH by XAS would be appropriate. However, PH samples residing exclusively in the Ni-B state have not been purified yet, most probably due to overlapping redox potential windows for Ni-B and other states (75, 96-97).

In agreement with previous findings (43, 45, 54), the inactive Ni-A state was not detected in any of the studied MBH samples, but Ni-A was the main species in the oxidized PH. Remarkably, the second oxygen atom of a bridging (hydro)peroxide (OOH⁻) proposed for Ni-A was not detectable by Ni-XAS, meaning that it was either absent or at a larger distance to Ni (Fig. 5B) than the ~2.2 Å derived from crystal structures of standard H₂ases (50, 87). A second O-atom could also be located on a terminal sulfenate group (SI-1).

MBH protein that was anaerobically oxidized prior to aerobic purification revealed the Ni-B state as the dominant site species. However, if this preparation was reduced with H₂ and subsequently reoxidized with air (45, 54), considerable amounts of the oxidatively modified Ni^{II}_{ia}-S species were found (Fig. 5B). A similar observation was made for MBH samples that were purified completely aerobically (43). According to the XAS and DFT results, this inactive state could feature a Ni-Fe bridging sulfenate group (Ni-O⁻S_{Cys}-Fe motif), besides of an additional Ni-bound oxygen species, possibly from OH⁻. A similar CysSO bridge has been detected in crystals of standard H₂ases (SI-1). Occurrence of the Ni_{ia}-S state in purified MBH protein may be related to the redox status and/or integrity of its FeS clusters (44-45). However, under physiological conditions, when the MBH is bound to the cytochrome-*b*

subunit in the membrane, the Ni_{ia}-S state has not been observed (43) and, hence, presumably is not of functional relevance.

Possible clues on the O₂ tolerance of the MBH of *R. eutropha*. The results in the present study support the previously made conclusion (11, 43, 45, 54) that a modified proximal FeS cluster, but not a modified [NiFe] site, is an essential prerequisite for O₂-tolerance in membrane-bound [NiFe] H₂ases. Likely, the key feature of the proximal cluster in the MBH is its altered redox potential compared to standard [NiFe] H₂ases (43, 46, 54), which facilitates additional electron donation to the active site. Redox transitions of this cluster are well detectable by EPR (43, 45). Oxidation of at least one additional Fe ion in the MBH compared to the PH is indicated by our Fe-XANES results. In the absence of external electron donors and starting in a situation with fully reduced iron-sulfur centers, the proximal FeS cluster, the medial [3Fe4S] cluster, and the distal [4Fe4S] cluster of the MBH thus can provide at least three electrons to the [NiFe] active site whereas only two electrons are provided by the distal [4Fe4S] and the medial [3Fe4S] clusters of the PH. This difference may explain, why the Ni-B state (Ni^{III}-OH⁻), which formally is by one electron more reduced than Ni-A (Ni^{III}-O⁻_{SCys},OH⁻ or Ni^{III}-OOH⁻), is preferred in the MBH. A surplus electron acceptor capacity due to the oxidized proximal cluster, in addition to the oxidized distal [4Fe4S] and medial [3Fe4S] clusters, on the other hand also is in agreement with the observed Ni-SR formation after activation by hydrogen of the MBH in the Ni-B state.

These considerations support a mechanism for H₂-cleavage by the MBH in an oxygen-containing environment, including only Ni-B and the intermediates in the catalytic cycle (Ni-S, -C, -SR) (54). During H₂-catalysis, the active MBH enzyme could provide electrons to reduce O₂ at the [NiFe] site in order to form the Ni-B state, which is rapidly reactivated. Thus, the catalytic activity is expected to depend on the relative concentrations of H₂ and O₂ and on the redox conditions controlling the availability of electrons (11). The absence of the Ni-A state is common to all three O₂-tolerant [NiFe] H₂ases of *R. eutropha* (24, 43, 85). Rapid O₂-

induced Ni-A formation and slow reactivation of this state in O₂-sensitive H₂ases is a major obstacle for their use in biotechnology. Thus, avoiding Ni-A formation is crucial for O₂-tolerant H₂ catalysis. Apparently, in the O₂-tolerant MBH, this goal mainly has been achieved by an enlarged reduction capacity for the active site due to a modified FeS cluster relay.

Acknowledgements

We thank Drs. W. Meyer-Klaucke (EMBL at DESY, Hamburg) and F. Schäfers and M. Mertin (beamline KMC-1 at BESSY, Helmholtz-Zentrum Berlin) for excellent technical support. M.H. thanks Prof. C. Limberg (HU-Berlin) for discussion and Dr. P. Chernev (FU-Berlin) for providing the GloFit program.

Supporting Information Available: (1) Sulfenates in [NiFe] H₂ase crystal structures, (2) XANES simulations, (3) DFT calculations on [NiFe] active site models, and references. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

References

1. (2007) Special Issue on Hydrogenases., *Chem. Rev.* 107.
2. Cammack, R. F., M.; Robson, R., (Ed.) (1997) *Hydrogen as a fuel: Learning from Nature*, Taylor & Francis, London, UK.
3. Rittmann, B. E. (2008) Opportunities for renewable bioenergy using microorganisms, *Biotechnol. Bioeng.* 100, 203-212.
4. Mertens, R., and Liese, A. (2004) Biotechnological applications of hydrogenases, *Curr. Opin. Biotechnol.* 15, 343-348.
5. Antoni, D., Zverlov, V. V., and Schwarz, W. H. (2007) Biofuels from microbes, *Appl. Microbiol. Biotechnol.* 77, 23-35.
6. Vincent, K. A., Parkin, A., Lenz, O., Albracht, S. P., Fontecilla-Camps, J. C., Cammack, R., Friedrich, B., and Armstrong, F. A. (2005) Electrochemical definitions of O₂ sensitivity and oxidative inactivation in hydrogenases, *J. Am. Chem. Soc.* 127, 18179-18189.
7. Burgdorf, T., Lenz, O., Buhrke, T., van der Linden, E., Jones, A. K., Albracht, S. P., and Friedrich, B. (2005) [NiFe]-hydrogenases of *Ralstonia eutropha* H16: modular enzymes for oxygen-tolerant biological hydrogen oxidation, *J. Mol. Microbiol. Biotechnol.* 10, 181-196.
8. Guiral, M., Aubert, C., and Giudici-Orticoni, M. T. (2005) Hydrogen metabolism in the hyperthermophilic bacterium *Aquifex aeolicus*, *Biochem. Soc. Trans.* 33, 22-24.
9. Yoon, K. S., Sakai, Y., Tsukada, N., Fujisawa, K., and Nishihara, H. (2009) Purification and biochemical characterization of a membrane-bound [NiFe]-hydrogenase from a hydrogen-oxidizing, lithotrophic bacterium, *Hydrogenophaga* sp. AH-24, *FEMS Microbiol. Lett.* 290, 114-120.
10. Parkin, A., Goldet, G., Cavazza, C., Fontecilla-Camps, J. C., and Armstrong, F. A. (2008) The difference a Se makes? Oxygen-tolerant hydrogen production by the [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum*, *J. Am. Chem. Soc.* 130, 13410-13416.
11. Lukey, M. J., Parkin, A., Roessler, M. M., Murphy, B. J., Harmer, J., Palmer, T., Sargent, F., and Armstrong, F. A. (2010) How *Escherichia coli* is equipped to oxidize hydrogen under different redox conditions, *J. Biol. Chem.* 285, 3928-3938.
12. Buhrke, T., Lenz, O., Krauss, N., and Friedrich, B. (2005) Oxygen tolerance of the H₂-sensing [NiFe] hydrogenase from *Ralstonia eutropha* H16 is based on limited access of oxygen to the active site, *J. Biol. Chem.* 280, 23791-23796.
13. Liebgott, P. P., de Lacey, A. L., Burlat, B., Cournac, L., Richaud, P., Brugna, M., Fernandez, V. M., Guigliarelli, B., Rousset, M., Leger, C., and Dementin, S. (2011) Original design of an oxygen-tolerant [NiFe] hydrogenase: major effect of a valine-to-cysteine mutation near the active site, *J. Am. Chem. Soc.* 133, 986-997.
14. Dementin, S., Leroux, F., Cournac, L., de Lacey, A. L., Volbeda, A., Leger, C., Burlat, B., Martinez, N., Champ, S., Martin, L., Sanganas, O., Haumann, M., Fernandez, V. M., Guigliarelli, B., Fontecilla-Camps, J. C., and Rousset, M. (2009) Introduction of methionines in the gas channel makes [NiFe] hydrogenase aero-tolerant, *J. Am. Chem. Soc.* 131, 10156-10164.
15. McTavish, H., Sayavedra-Soto, L. A., and Arp, D. J. (1995) Substitution of *Azotobacter vinelandii* hydrogenase small-subunit cysteines by serines can create insensitivity to inhibition by O₂ and preferentially damages H₂ oxidation over H₂ evolution, *J. Bacteriol.* 177, 3960-3964.
16. Canaguier, S., Artero, V., and Fontecave, M. (2008) Modelling NiFe hydrogenases: nickel-based electrocatalysts for hydrogen production, *Dalton Trans.* 315-325.
17. Rauchfuss, T. B. (2007) Chemistry. A promising mimic of hydrogenase activity, *Science* 316, 553-554.

18. Lu, Y. (2006) Biosynthetic inorganic chemistry, *Angew. Chem. Int. Ed. Engl.* 45, 5588-5601.
19. Bernhard, M., Buhrke, T., Bleijlevens, B., De Lacey, A. L., Fernandez, V. M., Albracht, S. P., and Friedrich, B. (2001) The H₂ sensor of *Ralstonia eutropha*. Biochemical characteristics, spectroscopic properties, and its interaction with a histidine protein kinase, *J. Biol. Chem.* 276, 15592-15597.
20. Lenz, O., Bernhard, M., Buhrke, T., Schwartz, E., and Friedrich, B. (2002) The hydrogen-sensing apparatus in *Ralstonia eutropha*, *J. Mol. Microbiol. Biotechnol.* 4, 255-262.
21. Buhrke, T., Loscher, S., Lenz, O., Schlodder, E., Zebger, I., Andersen, L. K., Hildebrandt, P., Meyer-Klaucke, W., Dau, H., Friedrich, B., and Haumann, M. (2005) Reduction of unusual iron-sulfur clusters in the H₂-sensing regulatory Ni-Fe hydrogenase from *Ralstonia eutropha* H16, *J. Biol. Chem.* 280, 19488-19495.
22. van der Linden, E., Burgdorf, T., de Lacey, A. L., Buhrke, T., Scholte, M., Fernandez, V. M., Friedrich, B., and Albracht, S. P. (2006) An improved purification procedure for the soluble [NiFe]-hydrogenase of *Ralstonia eutropha*: new insights into its (in)stability and spectroscopic properties, *J. Biol. Inorg. Chem.* 11, 247-260.
23. Burgdorf, T., Loscher, S., Liebisch, P., Van der Linden, E., Galander, M., Lenz, F., Meyer-Klaucke, W., Albracht, S. P., Friedrich, B., Dau, H., and Haumann, M. (2005) Structural and oxidation-state changes at its nonstandard Ni-Fe site during activation of the NAD-reducing hydrogenase from *Ralstonia eutropha* detected by X-ray absorption, EPR, and FTIR spectroscopy, *J. Am. Chem. Soc.* 127, 576-592.
24. Horch, M., Lauterbach, L., Saggi, M., Hildebrandt, P., Lenz, F., Bittl, R., Lenz, O., and Zebger, I. (2010) Probing the active site of an O₂-tolerant NAD⁺-reducing [NiFe]-hydrogenase from *Ralstonia eutropha* H16 by in situ EPR and FTIR spectroscopy, *Angew. Chem. Int. Ed. Engl.* 49, 8026-8029.
25. Lenz, O., Gleiche, A., Strack, A., and Friedrich, B. (2005) Requirements for heterologous production of a complex metalloenzyme: the membrane-bound [NiFe] hydrogenase, *J. Bacteriol.* 187, 6590-6595.
26. Ludwig, M., Cracknell, J. A., Vincent, K. A., Armstrong, F. A., and Lenz, O. (2009) Oxygen-tolerant H₂ oxidation by membrane-bound [NiFe] hydrogenases of *Ralstonia* species. Coping with low level H₂ in air, *J. Biol. Chem.* 284, 465-477.
27. Fontecilla-Camps, J. C., Volbeda, A., Cavazza, C., and Nicolet, Y. (2007) Structure/function relationships of [NiFe]- and [FeFe]-hydrogenases, *Chem. Rev.* 107, 4273-4303.
28. Armstrong, F. A., and Fontecilla-Camps, J. C. (2008) Biochemistry. A natural choice for activating hydrogen, *Science* 321, 498-499.
29. Ermler, U., Grabarse, W., Shima, S., Goubeaud, M., and Thauer, R. K. (1998) Active sites of transition-metal enzymes with a focus on nickel, *Curr. Opin. Struct. Biol.* 8, 749-758.
30. Peters, J. W. (1999) Structure and mechanism of iron-only hydrogenases, *Curr. Opin. Struct. Biol.* 9, 670-676.
31. Bernhard, M., Benelli, B., Hochkoeppler, A., Zannoni, D., and Friedrich, B. (1997) Functional and structural role of the cytochrome b subunit of the membrane-bound hydrogenase complex of *Alcaligenes eutrophus* H16, *Eur. J. Biochem.* 248, 179-186.
32. Friedrich, B., and Schwartz, E. (1993) Molecular biology of hydrogen utilization in aerobic chemolithotrophs, *Annu. Rev. Microbiol.* 47, 351-383.
33. Goldet, G., Wait, A. F., Cracknell, J. A., Vincent, K. A., Ludwig, M., Lenz, O., Friedrich, B., and Armstrong, F. A. (2008) Hydrogen production under aerobic conditions by membrane-bound hydrogenases from *Ralstonia* species, *J. Am. Chem. Soc.* 130, 11106-11113.

34. Cracknell, J. A., Vincent, K. A., Ludwig, M., Lenz, O., Friedrich, B., and Armstrong, F. A. (2008) Enzymatic oxidation of H₂ in atmospheric O₂: the electrochemistry of energy generation from trace H₂ by aerobic microorganisms, *J. Am. Chem. Soc.* 130, 424-425.
35. Vincent, K. A., Cracknell, J. A., Clark, J. R., Ludwig, M., Lenz, O., Friedrich, B., and Armstrong, F. A. (2006) Electricity from low-level H₂ in still air--an ultimate test for an oxygen tolerant hydrogenase, *Chem. Commun. (Camb.)*, 5033-5035.
36. Vincent, K. A., Cracknell, J. A., Lenz, O., Zebger, I., Friedrich, B., and Armstrong, F. A. (2005) Electrocatalytic hydrogen oxidation by an enzyme at high carbon monoxide or oxygen levels, *Proc. Natl. Acad. Sci. U. S. A.* 102, 16951-16954.
37. Fernandez, V. M., Hatchikian, E. C., and Cammack, R. (1985) Properties and reactivation of 2 different deactivated forms of *Desulfovibrio gigas* hydrogenase, *Biochim. Biophys. Acta* 832, 69-79.
38. Bleijlevens, B., van Broekhuizen, F. A., De Lacey, A. L., Roseboom, W., Fernandez, V. M., and Albracht, S. P. J. (2004) The activation of the [NiFe]-hydrogenase from *Allochromatium vinosum*. An infrared spectro-electrochemical study, *J. Biol. Inorg. Chem.* 9, 743-752.
39. Kortluke, C., Horstmann, K., Schwartz, E., Rohde, M., Binsack, R., and Friedrich, B. (1992) A gene complex coding for the membrane-bound hydrogenase of *Alcaligenes eutrophus* H16, *J. Bacteriol.* 174, 6277-6289.
40. Ludwig, M. (2008) *Ph.D. Thesis*, Humboldt Universität Berlin, Berlin, Germany.
41. Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1995) Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*, *Nature* 373, 580-587.
42. De Lacey, A. L., Pardo, A., Fernandez, V. M., Dementin, S., Adryanczyk-Perrier, G., Hatchikian, E. C., and Rousset, M. (2004) FTIR spectroelectrochemical study of the activation and inactivation processes of [NiFe] hydrogenases: effects of solvent isotope replacement and site-directed mutagenesis, *J. Biol. Inorg. Chem.* 9, 636-642.
43. Saggu, M., Zebger, I., Ludwig, M., Lenz, O., Friedrich, B., Hildebrandt, P., and Lenzian, F. (2009) Spectroscopic insights into the oxygen-tolerant membrane-associated [NiFe] hydrogenase of *Ralstonia eutropha* H16, *J. Biol. Chem.* 284, 16264-16276.
44. Saggu, M., Ludwig, M., Friedrich, B., Hildebrandt, P., Bittl, R., Lenzian, F., Lenz, O., and Zebger, I. (2010) Impact of amino acid substitutions near the catalytic site on the spectral properties of an O₂-tolerant membrane-bound [NiFe] hydrogenase, *Chemphyschem* 11, 1215-1224.
45. Saggu, M., Teutloff, C., Ludwig, M., Brecht, M., Pandelia, M. E., Lenz, O., Friedrich, B., Lubitz, W., Hildebrandt, P., Lenzian, F., and Bittl, R. (2010) Comparison of the membrane-bound [NiFe] hydrogenases from *R. eutropha* H16 and *D. vulgaris* Miyazaki F in the oxidized ready state by pulsed EPR, *PCCP* 12, 2139-2148.
46. Lenz, O., Ludwig, M., Schubert, T., Burstel, I., Ganskow, S., Goris, T., Schwarze, A., and Friedrich, B. (2010) H₂ conversion in the presence of O₂ as performed by the membrane-bound [NiFe]-hydrogenase of *Ralstonia eutropha*, *Chemphyschem* 11, 1107-1119.
47. Bernhard, M., Schwartz, E., Rietdorf, J., and Friedrich, B. (1996) The *Alcaligenes eutrophus* membrane-bound hydrogenase gene locus encodes functions involved in maturation and electron transport coupling, *J. Bacteriol.* 178, 4522-4529.
48. Schubert, T., Lenz, O., Krause, E., Volkmer, R., and Friedrich, B. (2007) Chaperones specific for the membrane-bound [NiFe]-hydrogenase interact with the Tat signal peptide of the small subunit precursor in *Ralstonia eutropha* H16, *Mol. Microbiol.* 66, 453-467.
49. Ludwig, M., Schubert, T., Zebger, I., Wisitruangsakul, N., Saggu, M., Strack, A., Lenz, O., Hildebrandt, P., and Friedrich, B. (2009) Concerted action of two novel auxiliary proteins in assembly of the active site in a membrane-bound [NiFe] hydrogenase, *J. Biol. Chem.* 284, 2159-2168.

50. Volbeda, A., Martin, L., Cavazza, C., Matho, M., Faber, B. W., Roseboom, W., Albracht, S. P. J., Garcin, E., Rousset, M., and Fontecilla-Camps, J. C. (2005) Structural differences between the ready and unready oxidized states of [NiFe] hydrogenases, *J. Biol. Inorg. Chem.* 10, 591-591.
51. Strange, R. W., and Feiters, M. C. (2008) Biological X-ray absorption spectroscopy (BioXAS): a valuable tool for the study of trace elements in the life sciences, *Curr. Opin. Struct. Biol.* 18, 609-616.
52. Dau, H., Liebisch, P., and Haumann, M. (2003) X-ray absorption spectroscopy to analyze nuclear geometry and electronic structure of biological metal centers - potential and questions examined with special focus on the tetra-nuclear manganese complex of oxygenic photosynthesis, *Anal. Bioanal. Chem.* 376, 562-583.
53. Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1995) Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*, *Nature* 373, 580-587.
54. Goris, T. W., Wait, A. F.; Saggu, M.; Fritsch, J.; Heidary, N.; Stein, M.; Zebger, I.; Lendzian, F.; Armstrong, F. A.; Friedrich, B.; Lenz, O. (2011) A unique iron-sulfur cluster plays a crucial role in oxygen tolerance of a [NiFe] hydrogenase, *Nat. Struct. Biol.* in press, doi:10.1038/nchembio.555.
55. Bradford, M. M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, *Anal. Biochem.* 72, 248-254.
56. Cammack, R., Fernandez, V. M., and Hatchikian, E. C. (1994) Nickel-iron hydrogenase, *Methods Enzymol.* 243, 43-68.
57. Klockenkämper, R. (1996) *Total Reflection X-ray Fluorescence Analysis*, Wiley-VCH, London, UK.
58. Loscher, S., Burgdorf, T., Zebger, I., Hildebrandt, P., Dau, H., Friedrich, B., and Haumann, M. (2006) Bias from H₂ cleavage to production and coordination changes at the Ni-Fe active site in the NAD⁺-reducing hydrogenase from *Ralstonia eutropha*, *Biochemistry* 45, 11658-11665.
59. Stripp, S., Sanganas, O., Happe, T., and Haumann, M. (2009) The structure of the active site H-cluster of [FeFe] hydrogenase from the green alga *Chlamydomonas reinhardtii* studied by X-ray absorption spectroscopy, *Biochemistry* 48, 5042-5049.
60. Gu, W., Jacquamet, L., Patil, D. S., Wang, H.-X., Evans, D. J., Smith, M. C., Millar, M., Koch, S., Eichhorn, D. M., Latimer, M., and Cramer, S. P. (2003) Refinement of the nickel site structure in *Desulfovibrio gigas* hydrogenase using range-extended EXAFS spectroscopy, *J. Inorg. Biochem.* 93, 41-51.
61. Pettifer, R. F., and Hermes, C. (1985) Absolute energy calibration of X-ray-radiation from synchrotron sources, *J. Appl. Crystallogr.* 18, 404-412.
62. Zabinsky, S. I., Rehr, J. J., Ankudinov, A. L., Albers, R. C., and Eller, M. J. (1995) Multiple-scattering calculations of x-ray-absorption spectra, *Phys. Rev. B* 52, 2995-3009.
63. Löscher, S., Schwartz, L., Stein, M., Ott, S., and Haumann, M. (2007) Facilitated hydride binding in an Fe-Fe hydrogenase active-site biomimic revealed by X-ray absorption spectroscopy and DFT calculations, *Inorg. Chem.* 46, 11094-11105.
64. Stein, M., and Lubitz, W. (2002) Quantum chemical calculations of [NiFe] hydrogenase, *Curr. Opin. Chem. Biol.* 6, 243-249.
65. Stein, M., and Lubitz, W. (2001) DFT calculations of the electronic structure of the paramagnetic states Ni-A, Ni-B and Ni-C of [NiFe] hydrogenase, *PCCP* 3, 2668-2675.
66. Neese, F. (2007) ORCA – An ab initio density functional and semiempirical program package (v. 2.6-35), Universität Bonn, Bonn, Germany.
67. Becke, A. D. (1988) Density-functional exchange-energy approximation with correct asymptotic behavior, *Phys. Rev. A* 38, 3098.

68. Perdew, J. P. (1986) Density-functional approximation for the correlation energy of the inhomogeneous electron gas, *Phys. Rev. B* 33, 8822.
69. Schäfer, A., Huber, C., and Ahlrichs, R. (1994) Fully optimized contracted Gaussian basis sets of triple zeta valence quality for atoms Li to Kr, *J. Chem. Phys.* 100, 5829-5835.
70. Andzelm, J., Kolmel, C., and Klamt, A. (1995) Incorporation of solvent effects into density-functional calculations of molecular energies and geometries, *J. Chem. Phys.* 103, 9312-9320.
71. Stephens, P. J., Devlin, F. J., Chabalowski, C. F., and Frisch, M. J. (1994) Ab-initio calculation of vibrational absorption and circular-dichroism spectra using density-functional force-fields, *J. Phys. Chem.* 98, 11623-11627.
72. Westre, T. E., Kennepohl, P., DeWitt, J. G., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1997) A multiplet analysis of Fe K-edge 1s->3d pre-edge features of iron complexes, *J. Am. Chem. Soc.* 119, 6297-6314.
73. Stiefel, E. I. G., G. N. (1994) Ferredoxins, hydrogenases, and nitrogenases: Metal-sulfide proteins., In *Bioinorg. Chem.* (Bertine, I. G., H. B.; Lippard, S. J.; Valentine, J. S., Eds.), pp 365-453, University Science Books, Mill Valley, USA.
74. Cammack, R., Fernandez, V. M., and Schneider, K. (1986) Activation and active-sites of nickel-containing hydrogenases, *Biochimie* 68, 85-91.
75. Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., Legall, J., Dervartanian, D. V., Peck, H. D., and Huynh, B. H. (1989) Redox intermediates of *Desulfovibrio gigas* [Nife] hydrogenase generated under hydrogen - Mössbauer and Epr characterization of the metal centers, *J. Biol. Chem.* 264, 16435-16450.
76. Carepo, M., Tierney, D. L., Brondino, C. D., Yang, T. C., Pamplona, A., Telser, J., Moura, I., Moura, J. J. G., and Hoffman, B. M. (2002) O-17 ENDOR detection of a solvent-derived Ni-(OHx)-Fe bridge that is lost upon activation of the hydrogenase from *Desulfovibrio gigas*, *J. Am. Chem. Soc.* 124, 281-286.
77. Brecht, M., Gastel, M. v., Buhrke, T., Friedrich, B., and Lubitz, W. (2003) Direct detection of a hydrogen ligand in the [NiFe] center of the regulatory H₂-sensing hydrogenase from *Ralstonia eutropha* in its reduced state by HYSCORE and ENDOR spectroscopy, *J. Am. Chem. Soc.* 125, 13075-13083.
78. Foerster, S., Stein, M., Brecht, M., Ogata, H., Higuchi, Y., and Lubitz, W. (2003) Single crystal EPR studies of the reduced active site of [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F, *J. Am. Chem. Soc.* 125, 83-93.
79. Teixeira, M., Moura, I., Xavier, A. V., Dervartanian, D. V., Legall, J., Peck, H. D., Huynh, B. H., and Moura, J. J. G. (1983) *Desulfovibrio gigas* hydrogenase - redox properties of the nickel and iron-sulfur centers, *Eur. J. Biochem.* 130, 481-484.
80. Niu, S., and Hall, N. B. (2001) Modeling the active sites in metalloenzymes 5. The heterolytic bond cleavage of H₂ in the [NiFe] hydrogenase of *Desulfovibrio gigas* by a nucleophilic addition mechanism, *Inorg. Chem.* 40, 6201-6203.
81. Wisitruangsakul, N., Lenz, O., Ludwig, M., Friedrich, B., Lendzian, F., Hildebrandt, P., and Zebger, I. (2009) Monitoring catalysis of the membrane-bound hydrogenase from *Ralstonia eutropha* H16 by surface-enhanced IR absorption spectroscopy, *Angew. Chem. Int. Edit.* 48, 611-613.
82. Colpas, G. J., Maroney, M. J., Bagyinka, C., Kumar, M., Willis, W. S., Suib, S. L., Mascharak, P. K., and Baidya, N. (1991) X-ray spectroscopic studies of nickel complexes, with application to the structure of nickel sites in hydrogenases, *Inorg. Chem.* 30, 920-928.
83. Davidson, G., Choudhury, S. B., Gu, Z., Bose, K., Roseboom, W., Albracht, S. P., and Maroney, M. J. (2000) Structural examination of the nickel site in *Chromatium vinosum* hydrogenase: redox state oscillations and structural changes accompanying reductive activation and CO binding, *Biochemistry* 39, 7468-7479.

84. Gu, Z. J., Dong, J., Allan, C. B., Choudhury, S. B., Franco, R., Moura, J. J. G., LeGall, J., Przybyla, A. E., Roseboom, W., Albracht, S. P. J., Axley, M. J., Scott, R. A., and Maroney, M. J. (1996) Structure of the Ni sites in hydrogenases by x-ray absorption spectroscopy. Species variation and the effects of redox poise, *J. Am. Chem. Soc.* **118**, 11155-11165.
85. Löscher, S., Gebler, A., Stein, M., Sanganas, O., Buhrke, T., Zebger, I., Dau, H., Friedrich, B., Lenz, O., and Haumann, M. (2010) Protein-protein complex formation affects the Ni-Fe and Fe-S centers in the H₂-sensing regulatory hydrogenase from *Ralstonia eutropha* H16, *ChemPhysChem* **11**, 1297-1306.
86. Leroux, F., Dementin, S., Burlatt, B., Cournac, L., Volbeda, A., Champ, S., Martin, L., Guigliarelli, B., Bertrand, P., Fontecilla-Camps, J., Rousset, M., and Leger, C. (2008) Experimental approaches to kinetics of gas diffusion in hydrogenase, *Proc. Natl. Acad. Sci. U. S. A.* **105**, 11188-11193.
87. Ogata, H., Hirota, S., Nakahara, A., Komori, H., Shibata, N., Kato, T., Kano, K., and Higuchi, Y. (2005) Activation process of [NiFe] hydrogenase elucidated by high-resolution X-ray analyses: Conversion of the ready to the unready state, *Structure* **13**, 1635-1642.
88. Higuchi, Y., Ogata, H., Miki, K., Yasuoka, N., and Yagi, T. (1999) Removal of the bridging ligand atom in the Ni-Fe active site of [NiFe] hydrogenase upon reduction with H₂, as revealed by X-ray structure analysis at 1.4 Å resolution, *Structure* **7**, 549-557.
89. Happe, R. P., Roseboom, W., Pierik, A. J., Albracht, S. P. J., and Bagley, K. A. (1997) Biological activation of hydrogen, *Nature* **385**, 126-126.
90. Beinert, H., Holm, R. H., and Munck, E. (1997) Iron-sulfur clusters: Nature's modular, multipurpose structures, *Science* **277**, 653-659.
91. Knüttel, K. S., K.; Erkens, A.; Plass, W.; Müller, A.; Bill, E.; Trautwein, A. X. (1994) Redox properties of the metal centers in membrane-bound hydrogenase from *Alcaligenes eutrophus*., *Bull. Pol. Acad. Sci., Chem.* **42**, 495-511.
92. Pandelia, M. E., Fourmond, V., Tron-Infossi, P., Lojou, E., Bertrand, P., Leger, C., Giudici-Orticoni, M. T., and Lubitz, W. (2010) Membrane-bound hydrogenase I from the hyperthermophilic bacterium *Aquifex aeolicus*: enzyme activation, redox intermediates and oxygen tolerance, *J. Am. Chem. Soc.* **132**, 6991-7004.
93. Ogata, H., Kellers, P., and Lubitz, W. (2010) The Crystal structure of the [NiFe] hydrogenase from the photosynthetic bacterium *Allochromatium vinosum*: characterization of the oxidized enzyme (Ni-A state), *J. Mol. Biol.* **402**, 428-444.
94. Matias, P. M., Soares, C. M., Saraiva, L. M., Coelho, R., Morais, J., Le Gall, J., and Carrondo, M. A. (2001) [NiFe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 27774: gene sequencing, three-dimensional structure determination and refinement at 1.8 angstrom and modelling studies of its interaction with the tetrahaem cytochrome c(3), *J. Biol. Inorg. Chem.* **6**, 63-81.
95. Pandelia, M. E., Infossi, P., Giudici-Orticoni, M. T., and Lubitz, W. (2010) The oxygen-tolerant hydrogenase I from *Aquifex aeolicus* weakly interacts with carbon monoxide: An electrochemical and time-resolved FTIR study, *Biochemistry* **49**, 8873-8881.
96. Roberts, L. M., and Lindahl, P. A. (1994) Analysis of oxidative titrations of *Desulfovibrio gigas* hydrogenase; implications for the catalytic mechanism, *Biochemistry* **33**, 14339-14350.
97. Dole, F., Fournel, A., Magro, V., Hatchikian, E. C., Bertrand, P., and Guigliarelli, B. (1997) Nature and electronic structure of the Ni-X dinuclear center of *Desulfovibrio gigas* hydrogenase. Implications for the enzymatic mechanism, *Biochemistry* **36**, 7847-7854.

Table 1

sample	fit	shell	N _i [per Fe]	R _i [Å]	2σ _i ² x10 ³ [Å ²]	R _F [%]
PH ox	I (II)	Fe-C	0.33 [*] (0.44)	2.08 (2.07)	2 (2 [*])	9.8 (8.5)
		Fe-S	3.83 [*] (3.63)	2.28 (2.28)	7 (6)	
		Fe-Fe	2.58 [*] (2.49)	2.72 (2.72)	7 (8)	
MBH ox	III (IV)	Fe-O	0.33 [*] (0.57)	2.05 (2.06)	2 [*] (2 [*])	18.0 (11.8)
		Fe-S	3.83 [*] (3.17)	2.28 (2.29)	9 (7 [*])	
		Fe-Fe	2.58 [*] (1.50)	2.72 (2.74)	14 (7 [*])	
	V (VI)	Fe-O	0.58 (0.57)	2.06 (2.06)	2 [*] (2 [*])	8.2 (8.9)
		Fe-S	3.37 (3.37)	2.29 (2.29)	7 [*] (7 ⁺)	
		Fe-S	0.82 (0.91)	2.60 (2.61)	7 [*] (7 [*])	
		Fe-Fe	1.85 (1.24)	2.72 (2.66)	7 [*] (7 [*])	
		Fe-Fe	0.35 (0.88)	3.34 (2.79)	7 [*] (7 [*])	
MBH H ₂	VII (VIII)	Fe-O	0.35 (0.43)	2.13 (2.13)	2 [*] (2 [*])	11.1 (12.3)
		Fe-S	3.78 (3.73)	2.31 (2.31)	7 [*] (7 [*])	
		Fe-S	0.78 (0.26)	2.73 (2.60)	7 [*] (7 [*])	
		Fe-Fe	1.81 (1.08)	2.74 (2.69)	7 [*] (7 [*])	
		Fe-Fe	0.55 (0.93)	3.42 (2.80)	7 [*] (7 [*])	
MBH C19G/C120G H ₂	IX	Fe-O	0.48	2.07	2 [*]	12.5
		Fe-S	3.68	2.28	7 [*]	
		Fe-S	0.83	2.59	7 [*]	
		Fe-Fe	2.36	2.72	7 [*]	
		Fe-Fe	0.25	3.02	7 [*]	

Table 2

state of [NiFe] site	Ni _r -B	Ni _{ia} -S	Ni-S (Ni _r -S, Ni _{r/u} -S)	Ni _a -C	Ni _a -SR (Ni _a -SR, -SR', -SR'')
Ni oxidation state	III	II	II	III	II
ν(CO) [cm ⁻¹]	1948.0	1929.6	1933.8, 1944.7	1957.0	1944.7, 1926.2, 1918.0
oxidized, pH 5.5	71	0	28 (13, 15)	1 ^(e)	0
H ₂ -reduced, pH 5.5	0	0	11 (11, 0)	10	79 (18, 50, 11)
air-reoxidized, pH 5.5	36 ^(a)	20	41 (13, 28)	3 ^(e)	0
oxidized, pH 8.0 ^(d)	2 ^(b)	69	26 (13 ^(c) , 13)	3 ^(e)	0

Table 3

	shell:	Ni-O	Ni-S	Ni-Fe	
sample:	fit:	N _i [per Ni] / R _i [Å] / 2σ ² x10 ³ [Å ²]			R _F [%]
PH					
Ni-A	I	0.82 / 1.85 / 4 [*]	2.73 [#] / 2.16 / 5 1.27 [#] / 2.72 / 4 [*]	1.00 [*] / 2.75 / 5 [*]	11.2
Ni-C	II	0.13 / 1.83 / 4 [*]	3.03 [#] / 2.18 / 7 0.97 [#] / 2.55 / 4 [*]	1.00 [*] / 2.54 / 5 [*]	15.3
Ni-SR	III	0.02 / 1.80 / 4 [*]	3.23 [#] / 2.17 / 9 0.77 [#] / 2.56 / 4 [*]	1.00 [*] / 2.51 / 5 [*]	17.3
MBH					
Ni-B	IV	0.67 / 1.88 / 4 [*]	2.71 [#] / 2.20 / 14 1.29 [#] / 2.62 / 4 [*]	1.00 [*] / 2.64 / 5 [*]	12.1
Ni-SR	V	0.12 / 1.80 / 4 [*]	3.07 [#] / 2.21 / 6 0.93 [#] / 2.60 / 4 [*]	1.00 [*] / 2.58 / 5 [*]	12.8
Ni _{ia} -S	VI	1.41 / 2.03 / 4 [*]	2.30 [#] / 2.32 / 22 1.70 [#] / 2.98 / 4 [*]	1.00 [*] / 3.05 / 5 [*]	12.5

Legends to Tables and Figures

Table 1: Simulation parameters of Fe EXAFS spectra of PH and MBH. C19G/C120G denotes a HoxK variant of the MBH (54); ox = oxidized and H₂ = hydrogen-reduced proteins. N_i , coordination number; R_i , Fe-backscatterer distance; $2\sigma_i^2$, Debye-Waller factor. (*) Fixed parameters in the fit procedure. For fit I, the N_i -values for the PH were calculated including (i) a set of 2x [4Fe4S] and 1x [3Fe4S] clusters (the distal [4Fe4S] cluster is ligated by one N-atom from a histidine), (ii) one Fe-Ni vector which was indistinguishable from the Fe-Fe distances, and (iii) coordination of the active-site Fe by two CysS and three carbons from the two CN⁻ ligands and the CO. The R_F -value (48) was calculated for reduced distances of 1-3 Å.

Table 2: Redox-state populations of the [NiFe] site in various MBH preparations. Populations (in %, error of about ±5 %) were derived from Gaussian fits of the CO stretching vibrations in the FTIR absorbance spectra (not shown, see (43-44)). The respective state assignments follow previous attributions (43). Indices denote: a, active; ia, inactive; r, ready; and u, unready states; the assignment of the 1944.7 cm⁻¹ band is ambiguous, however, it belongs presumably to an oxidized EPR-silent state. ^(a)Band at 1950.6 cm⁻¹, ^(b)1948.9 cm⁻¹, and ^(c)1935.9 cm⁻¹. ^(d)Values for the less active MBH preparation. ^(e)These minor contributions are presumably due to a further inactive Ni-S state.

Table 3: Ni EXAFS simulation parameters for PH and MBH. State nominations follow attributions in Table 2. N_i , coordination number; R_i , Ni-backscatterer distance; $2\sigma_i^2$, Debye-Waller factor. *Fixed parameters in the fit procedure, #the sum of the N_{Ni-S} values was restrained to 4. R_F was calculated for reduced distances of 1-2.5 Å.

Figure 1: Composition of [NiFe] hydrogenases of the standard type (PH) from *D. gigas* (53) and of the membrane-bound type (MBH) from *R. eutropha* (25, 31). Two CN⁻ and one CO at the active site iron were inferred by FTIR (43). The enzyme preparations lacked the membrane and the cytochrome-*b* subunit with its two heme groups in the case of MBH and the cytochrome-*c* in the case of PH. FeS denotes a modified proximal cluster in the MBH.

Figure 2: Fe XAS spectra. (A) Fourier-transforms (FTs) of EXAFS spectra (in B) of oxidized (red) and H₂-reduced (blue) MBH, of the HoxK C19G/C120G mutant of MBH (magenta), and of oxidized PH (green). The bar marks the difference between FT-peak amplitudes due to Fe-Fe vectors in wildtype MBH and PH. Inset: FTs of MBH at pH 5.5 (line) and of the less active MBH preparation at pH 8 (dots). FTs were calculated for a *k*-range of 2-16 Å⁻¹ and using cos² windows over 10 % at both *k*-range ends. (B) Experimental EXAFS spectra (black) and simulations (coloured lines) with parameters in Table 1 (fits I, VI, VIII, IX). (C) Respective Fe K-edge spectra and their pre-edge features on an expanded energy scale in the inset.

Figure 3: Ni K-edge spectra of MBH and PH samples containing mostly the indicated states of the [NiFe] site with formal Ni(III) (Ni-A, -B, -C) or Ni(II) (Ni-SR, Ni_{ia}-S) oxidation states. The spectrum of an air-reoxidized MBH sample is shown as a dashed line. Spectra of MBH were vertically displaced. The inset shows K-edge energies at 50 % level of edge spectra.

Figure 4: Ni EXAFS spectra. (A) FTs of spectra of PH in the oxidized (Ni-A), H₂-reduced (Ni-C), and Na-dithionite reduced (Ni-SR) states. (B) FTs of spectra of MBH in the oxidized (Ni-B), H₂-reduced (Ni-SR), and inactive (Ni_{ia}-S) states. The arrows mark FT-peaks due to Ni-μO bonds; * contributions from Ni-Fe vectors. FTs were calculated for a *k*-range of 2-15 Å⁻¹ and using cos² windows over 10 % at both *k*-range ends. Insets in (A) and (B) show the

respective experimental EXAFS spectra (thin black lines) and their simulations (thick lines) with parameters in Table 3. All spectra are displayed on the same scale.

Figure 5: Structural motifs of FeS clusters and the [NiFe] site in PH and MBH. (A) Left: structure of the proximal [4Fe4S] cluster in crystallized PH (53). Right: hypothetical structure of the proximal FeS cluster in MBH in agreement with the Fe-XAS results. The model was constructed from the PH structure by moving one iron (Fe_d) to $\sim 3.4 \text{ \AA}$ from two other Fe ions. The particular positions of the two additional cysteines (C19, C120) are plausible according to modelling results (54). A bridging CysS may account for the shortest Fe-Fe distance of $\sim 2.6 \text{ \AA}$. The connections of Fe_d to the other Fe ions by μS bonds and the presence of four such motifs is unclear (dotted lines). (B) Model structures of the [NiFe] site in agreement with the Ni-XAS results. The S-atoms belong to cysteines; rounded Ni-Fe and first-sphere Ni-oxygen distances (in $\text{\AA}$) are indicated; the iron is in the Fe(II) state. The Ni-B state is assigned to a bridging hydroxide (54). The Ni-A state may be assigned either to a bridging OH^- plus a terminal CysSO or to a bridging hydroperoxide (50, 88, 89), with the non-bridging O-atoms relatively distant from Ni and not detectable by XAS. The location of the bridging CysSO in $\text{Ni}_{ia}\text{-S}$ follows crystallographic data of standard H_2ases (SI-1), the Ni-bound OH^- is tentative. Possible Ni-bound H-species in Ni-SR were omitted.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supporting Information

[NiFe]- and [FeS]-cofactors in the membrane-bound hydrogenase of Ralstonia eutropha investigated by X-ray absorption spectroscopy: insights into O₂-tolerant H₂-cleavage

Johannes Fritsch¹, Simone Löscher², Oliver Sanganas², Elisabeth Siebert³, Ingo Zebger³, Matthias Stein⁴, Marcus Ludwig¹, Antonio L. De Lacey⁵, Holger Dau², Bärbel Friedrich¹, Oliver Lenz¹, and Michael Haumann²

¹Humboldt-Universität zu Berlin, Institut für Biologie/Mikrobiologie, 10115 Berlin, Germany

²Freie Universität Berlin, Institut für Experimentalphysik, 14195 Berlin, Germany

³Technische Universität Berlin, Max-Volmer Institut, 10623 Berlin, Germany

⁴Max-Planck-Institut für Dynamik komplexer technischer Systeme, 39106 Magdeburg, Germany

⁵Instituto de Catálisis y Petroleoquímica (CSIC), 28049 Madrid, Spain

(1) Sulfenates in [NiFe] H₂ase crystal structures.

In several crystal structures of hydrogenases in the PDB database, sulfenates (CysSO) at the Ni-Fe active site were observed (Fig. S1). Sulfenates have been found in samples predominantly containing the Ni-A, Ni-B, and even reduced states of H₂ase proteins. In two structures (1YQW, 1WUI) an equatorial bridging sulfenate bound via its O-atom to Ni was found (Fig. S1, top), similar to what we propose for the inactive Ni_{ia}-S state of MBH. Evidence for additional oxygen ligands at Ni has also been obtained in previous XAS investigations on various H₂ases (1-4).

Figure S1: Sulfenate formation of cysteine residues (CysSO) in crystal structures of Ni-Fe H₂ases (respective PDB entry numbers are indicated). Structures have been derived for H₂ase from *D. gigas* (1YQW), *D. vulgaris* Miyazaki F (1WUI, 1WUJ, 1WUL), and from a methionine double-mutant of *D. fructosovorans* (3CUS). Colour code of elements: Green, Ni; magenta, Fe; red, O; blue, N; cyan, C; yellow, S. Structures are anticipated to correspond to the Ni-A state (top), to Ni-B (middle), and to reduced proteins presumably mainly in the Ni-SR state (bottom). Possible protons on the bridging oxygen species are invisible in the crystal structures.

(2) XANES simulations.

Calculations of XANES spectra were performed on [NiFe] site models (5) similar to those shown in Fig. 5. The calculated spectra reproduced the spectral changes, i.e. a decreased secondary edge maximum, for the transition from a 5-coordinated Ni(III) in Ni-B ((Cys)₄Ni^{III}μO(H)) to a 4-coordinated Ni^{II} (which may contain an additional, but XAS invisible H-ligand) in Ni-SR ((Cys)₄Ni^{II}) (Fig. S2). The large primary edge maximum of the Ni_{ia}-S state of MBH was only reproduced including two Ni-bound oxygen species, i.e. a metal-bridging CysSO group and a terminal hydroxide ligand (compare Fig. S2 and Fig. 5); a single terminal OH was insufficient.

Figure S2: Calculated XANES spectra based on the following Ni-coordinations: (Cys)₄Ni-μOH, line; (Cys)₄Ni, ●; (Cys)₄Ni-OH, ○; (Cys)₃(CysS)ONi-OH, dash-dots. The horizontal line marks the unity level. For structures containing CysSO groups see SI-1.

(3) DFT calculations on [NiFe] active site models.

Density-functional theory calculations were carried out on [NiFe] site models similar to those in Fig. 5 for the Ni^{III}-B and Ni^{II}-S states, involving oxidized cysteines (CysSO) either in a metal-bridging position or in a terminal position (see Fig. S1). The results (Table S1) support the presence of a Ni-Fe bridging CysSO (O-atom bound to Ni, S-atom at ~2.9 Å to Ni) in Ni_{ia}-S of MBH. Only small parameter differences were observed for terminal CysSO groups, bound to Ni via their S-atom. For further details see the Results section.

Table S1: Structural parameters of [NiFe] site models from DFT.

		Ni ^{III} -B (Ni ^{II} -S)
exp. Ni-Fe distance [Å]	PH	2.84 ^(a)
	MBH	2.65 (3.05)
calc. Ni-Fe distance [Å]	(CysS) ₄	2.94 (2.63)
	(CysS) ₃ (CysS)O ⁶⁸ _{bridge}	-3.12(3.01)
	(CysS) ₃ (CysS)O ⁶⁵ _{terminal}	2.89 (2.64)
exp. Ni-S distance [Å]	PH	2.31 ^(b)
	MBH	2.34 (2.60)
calc. average Ni-S distance [Å]	(CysS) ₄	2.28 (2.34)
	(CysS) ₃ (CysS)O ⁶⁸ _{bridge}	2.49 (2.57)
	(CysS) ₃ (CysS)O ⁶⁵ _{terminal}	2.30 (2.33)
exp. Ni-O distance [Å]	PH	1.67 ^(b)
	MBH	1.88 (2.03)
calc. Ni-OH distance [Å]	(CysS) ₄	1.94
	(CysS) ₃ (CysS)O ⁶⁸ _{bridge}	1.93 (1.97)
	(CysS) ₃ (CysS)O ⁶⁵ _{terminal}	1.91 (1.95)
calc. Ni-OSCys distance [Å]	(CysS) ₄	none
	(CysS) ₃ (CysS)O ⁶⁸ _{bridge}	2.03 (1.90)
	(CysS) ₃ (CysS)O ⁶⁵ _{terminal}	3.12 (3.19)

Experimental data from this work and the literature for PH and MBH are given for comparison; values in parenthesis are for the Ni_{ia}-S state of MBH. Numbering of Cys ligands follows the *D. gigas* notation. In parenthesis, values for Ni-S. ^(a)Data from (6); ^(b)data from structure 1WUJ of *D. vulgaris* Miyazaki F H₂ase.

References

1. Maroney, M. J., Colpas, G. J., Bagyinka, C., Baidya, N., and Mascharak, P. K. (1991) Exafs investigations of the Ni site in *Thiocapsa roseopersicina* hydrogenase - Evidence for a novel Ni,Fe,S cluster, *J. Am. Chem. Soc.* 113, 3962-3972.
2. Kumar, M., Colpas, G. J., Day, R. O., and Maroney, M. J. (1989) Ligand oxidation in a nickel thiolate complex - a model for the deactivation of hydrogenase by O₂, *J. Am. Chem. Soc.* 111, 8323-8325.
3. Maroney, M. J. (1999) Structure/function relationships in nickel metallobiochemistry, *Curr. Opin. Chem. Biol.* 3, 188-199.
4. Maroney, M. J., Colpas, G. J., and Bagyinka, C. (1990) X-Ray absorption spectroscopic structural investigations of the Ni site in reduced *Thiocapsa roseopersicina* Hydrogenase, *J. Am. Chem. Soc.* 112, 7067-7068.
5. Burgdorf, T., Loscher, S., Liebisch, P., Van der Linden, E., Galander, M., Lendzian, F., Meyer-Klaucke, W., Albracht, S. P., Friedrich, B., Dau, H., and Haumann, M. (2005) Structural and oxidation-state changes at its nonstandard Ni-Fe site during activation of the NAD-reducing hydrogenase from *Ralstonia eutropha* detected by X-ray absorption, EPR, and FTIR spectroscopy, *J. Am. Chem. Soc.* 127, 576-592.
6. Davidson, G., Choudhury, S. B., Gu, Z., Bose, K., Roseboom, W., Albracht, S. P., and Maroney, M. J. (2000) Structural examination of the nickel site in *Chromatium vinosum* hydrogenase: redox state oscillations and structural changes accompanying reductive activation and CO binding. *Biochemistry* 39, 7468-7479.