The F₄₂₀-reducing [NiFe]-hydrogenase complex from *Methanothermobacter marburgensis*, the first X-ray structure of a group 3 family member

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Abstract

The reversible redox reaction between coenzyme F_{420} and H_2 to $F_{420}H_2$ is catalyzed by a F_{420} -reducing [NiFe]-hydrogenase (FrhABG) which is an enzyme of the energy metabolism of methanogenic archaea. FrhABG is a group 3 [NiFe]-hydrogenase with a dodecameric quaternary structure of 1.25 MDa as recently revealed by high resolution cryo electron microscopy. We report on the crystal structure of FrhABG from *Methanothermobacter marburgensis* at 1.7 Å resolution and compare it with the structures of group 1 [NiFe]-hydrogenases, the only group structurally characterized yet. FrhA is similar to the large subunit of group 1 [NiFe]-hydrogenases regarding its core structure and the embedded [NiFe]-center but different because of the truncation of ca. 160 residues which results in similar but modified H_2 - and proton- transport pathways and in suitable interfaces for oligomerization. The small subunit FrhG is composed of a N-terminal domain related to group 1 enzymes and a new C-terminal ferredoxin-like domain carrying the distal and medial [4Fe-4S] clusters. FrhB adopts a novel fold, binds one [4Fe-4S] cluster as well as one FAD in a U-shaped conformation and provides the F_{420} -binding site at the *Si*-face of the isoalloxazine ring. Similar electrochemical potentials of both catalytic reactions and the electron-transferring [4Fe-4S] clusters, determined to be $E^{\circ'} \approx -400$ mV, are in full agreement with the equalized forward and backward rates of the FrhABG reaction. The protein might contribute to balanced redox potentials by the aspartate coordination of the proximal [4Fe-4S] cluster, the new ferredoxin module and a rather negatively charged FAD surrounding.

Introduction

[NiFe]-hydrogenases are classified into five groups.^{1; 2} All known crystal structures belong to group 1 [NiFe]-hydrogenases which are built up of a large and a small subunit. The large subunit hosts the [NiFe]-center, the site of H₂ activation.³ The small subunit mostly contains a proximal [4Fe-4S], a medial [3Fe-4S] and a distal [4Fe-4S] cluster. Electrons released upon H₂ oxidation at the [NiFe]-center are transferred via the proximal and medial to the distal Fe/S cluster and further to an external cytochrome *b* or *c*. The proximal [4Fe-4S] cluster 6 Å apart from the [NiFe]-center appears to be crucial for H₂ activation.⁴ Recently, it was discovered that a special proximal [4Fe-3S] cluster is responsible for O₂ stability in oxygen-tolerant [NiFe]-hydrogenases.^{5; 6; 7}

In the energy metabolism of hydrogenotrophic methanogenic archaea H₂ and CO₂ are used for methane formation ($4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$). The four H₂ molecules are activated by three types of [NiFe]- and one [Fe]-hydrogenases.⁸ Four from eight electrons for CO₂ reduction are supplied by reduced coenzyme F₄₂₀ which is regenerated by H₂ oxidation ($\text{H}_2 + \text{F}_{420} \rightleftharpoons \text{F}_{420}\text{H}_2$; $\Delta G^{\circ'} = -10 \text{ kJ/mol}$) via a F₄₂₀-reducing [NiFe]-hydrogenase (FrhABG).⁹ F₄₂₀ is a hydride-transferring 5'-deazaflavin that functions like NAD(P).¹⁰ The FrhABG reaction is reversible and *in vitro* data indicate the same forward and backward rate.^{11; 12} In hydrogenotrophic methanogenesis, FrhABG is exclusively used for F₄₂₀ reduction and in methanogens growing on formate or C₁ compounds also for H₂ production.^{13; 14}

FrhABG, encoded by the transcriptional unit *frhADGB*, is a member of the group 3 [NiFe]-hydrogenases. The large and small subunits FrhA (43 kDa) and FrhG (25 kDa) encoded by *frhA* and *frhG* carry the [NiFe]-center and three [4Fe-4S] clusters, respectively. The primary structure of FrhG differs from that of group 1 [NiFe]-hydrogenases in a manner that only the N-terminal is conserved but not the C-terminal domain.¹⁵ The C-terminal domain consists of a ferredoxin module with two [4Fe-4S] clusters.¹⁶ Notably, one of the conserved cysteines coordinating the proximal [4Fe-4S] cluster of the N-terminal domain in group 1 enzymes appears to be replaced by an aspartate in FrhG. FrhB (31 kDa), absent in group 1 [NiFe]-hydrogenases, harbors one [4Fe-4S] cluster, one FAD and the F₄₂₀-

binding site. The *frhD* gene encodes a maturing endopeptidase, which probably cleaves off the C-terminal end of FrhA during maturation.¹⁷

The overall architecture of FrhABG of *Methanothermobacter marburgensis* has recently been analyzed by Cryo-EM analysis¹⁸ which revealed a huge protein complex composed of twelve FrhABG heterotrimers arranged as a cube with a solvent-filled cavity. The Cryo-EM structure largely allowed model building of the polypeptide backbone and the detection of the cofactors (pdb code: 3ZF5). After submitting our work a more accurate Cryo-EM structure has been published.¹⁹ In this report, we present the first crystal structure of a group 3, archaeal and cytosolic [NiFe]-hydrogenase. The 1.7 Å electron density map of FrhABG from *M. marburgensis* revealed the structures of FrhA, FrhB and FrhG, the oligomerization sites, the metal cofactors and the FAD binding mode in great detail. Moreover, the structural data offer valuable information about H₂ channels, proton relay pathways, F₄₂₀ binding and the reversibility of the catalyzed reaction.

Results and discussion

Purification and characterization of the FrhABG complex

Cell disruption and purification of FrhABG was performed in a 95% N₂/ 5% H₂ atmosphere without adding oxidizing and reducing agents. The resulting state is termed “as isolated” FrhABG. Due to its size of 1.25 MDa FrhABG from *M. marburgensis* has co-sedimented with the membrane fraction upon ultracentrifugation at 100,000 ´ g. Therefore, the centrifugal speed force has to be reduced to 15,000 ´ g. The dodecameric FrhABG complex tends to aggregate with FrhABG sub-complexes and other proteins. It was found out that incubation of the sample with freshly prepared sodium dithionite (4 mM) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, 0.6 mg per mg protein) followed by anion-exchange and size exclusion chromatographic steps provided a homogeneous FrhABG complex as demonstrated by SDS-PAGE (Fig. S1) and Cryo-EM.¹⁸ The presence of both compounds did not affect the F₄₂₀-reducing activity. The specific activity obtained was 280 U/mg (470 s⁻¹), at 70 °C under 100% H₂ which was within the normal activity range

found for [NiFe]-hydrogenases.²⁰ The apparent K_m values were 35 μM for F_{420} at 100% H_2 in the gas phase and 8% for H_2 in the gas phase at 40 μM F_{420} concentration.

FrhB from *Methanocaldococcus jannaschii* was heterologously overproduced in *Escherichia coli* and purified (Fig. S2). Recombinant FrhB reduces benzyl viologen with $F_{420}\text{H}_2$ under anaerobic conditions (Fig. S3). In the assay, $F_{420}\text{H}_2$ was regenerated by hydride transfer from methylenetetrahydromethanopterin catalyzed by F_{420} -dependent methylenetetrahydromethanopterin dehydrogenase.²¹ The apparent $V_{\max}$ was ca. 12 U/mg with an apparent K_M for $F_{420}\text{H}_2$ of $12 \pm 2 \mu\text{M}$ (Fig. S3). These data indicated that FrhB binds and oxidizes $F_{420}\text{H}_2$.

Redox titration

The redox potentials of the [4Fe-4S] clusters of the FrhABG complex and recombinant FrhB were determined by redox titration in the range -433 – +37 mV and -455 – +3 mV, respectively, using potassium hexacyanoferrate and sodium dithionite as oxidizing and reducing agents, respectively. The redox states of the [4Fe-4S] clusters were followed by EPR spectroscopy in the presence of the mediators benzyl- and methylviologen. Although the midpoint potentials of the individual clusters could not be accurately determined, their estimation was possible. In our measurements, reduced FrhABG exhibited a well-defined rhombic EPR signal for one $[4\text{Fe-4S}]^{1+}$ cluster with g -values of $g_z = 2.059$, $g_y = 1.961$ and $g_x = 1.888$, and a broader signal with $g = 1.933$ from a second $[4\text{Fe-4S}]^{1+}$ center. The rhombic EPR signal belonging to one of the reduced $[4\text{Fe-4S}]^{1+}$ clusters developed below -360 mV with an estimated midpoint potential of -400 mV (Fig. S4). In comparison, the redox potential of the F_{420} -reducing [NiFeSe]-hydrogenase from *Methanococcus voltae* was determined to -370 mV.²² The redox potential of the [4Fe-4S] cluster of the heterologously expressed FrhB measured by the same procedure from the intensity of the rather featureless axial EPR signal was approximately -450 mV, which is slightly lower than the redox midpoint potential of the [4Fe-4S] cluster giving rise to the rhombic EPR signal in the FrhABG complex.

Crystallization

Crystallization succeeded under several conditions using polyethylene glycol (PEG) as a precipitant, however, crystals suitable for X-ray structure analysis only grew in the presence of the additive trimethyl-N-oxide. The cubic brownish crystals grew as twins and diffracted up to 1.7 Å resolution (Fig. S1 and Table S1). The unit cell contains two FrhABG dodecamers and the asymmetric unit contains one FrhABG heterotrimer because the symmetry of the space group $F23$ and of the FrhABG dodecamer corresponds to each other. Phase determination was hampered by the substantial radiation damage and the varying twinning fraction between different crystals and within one crystal; consequently data measured at different crystal positions could not be merged. A reasonable electron density was obtained after combining the phases from SAD data (Table S1) and from the Cryo-EM density map.¹⁸ The refined structure of the “as isolated” FrhABG is based on a crystal part with a very low twinning fraction. The final R_{work} and R_{free} factors are 15.0 and 18.1%, respectively, at 1.7 Å resolution. The crystal structure of FrhABG oxidized with potassium hexacyanoferrate was also determined (Table S1) but its resolution at 2.6 Å is not sufficient for detecting small differences to the “as isolated” structure.

Structure of the FrhABG heterotrimer

The FrhABG heterotrimer represents the protomer of the dodecameric FrhABG complex adopting an oval shape with FrhA and FrhB at both ends and FrhG in between (Fig. 1). FrhA, FrhB and FrhG are tightly associated burying a surface area of ca. 4900 Å² (Fig. 1a, Table S2).

FrhA: The structure of the large subunit FrhA (1-386) can be subdivided into four tightly associated domains (Fig. 2a). Terminal domain I (1-39, 350-386) is composed of an N-terminal β -meander faced by two C-terminal helices and terminal domain II (300-349, 40-86), in reverse, of a C-terminal β -meander and three N-terminal helices. The intermediate part consists of a four-helix bundle domain (87-195; 276-300) interrupted after the third α -helix (165:195) by the insertion domain (196-275). The insertion domain attached to terminal domains I and II consists of two short two-stranded β -sheets, a few short helices and, in particular, of elements without secondary structures. Although structurally related to group 1 [NiFe]-hydrogenases reflected in a low root-mean-square deviation

(rmsd) of ca. 2.0 Å (calculated for the most related *Desulfovibrio gigas* and *Desulfovibrio vulgaris* enzymes^{3; 23} with 70% and 77% of the C_α atoms), FrhA is ca. 160 residues shorter. Accordingly, 80 residues were cut in the loop between helices 90:114 and 123:143 of the four-helix bundle domain, 60 residues in the insertion domain, five and seven residues in the loops after helices 276:298 (four-helix bundle domain) and 350:362 (terminal domain I), respectively and 10 residues in a protrusion inserted within helix 350:362 (terminal domain I). The structurally characterized [NiFeSe]-hydrogenase of *D. vulgaris* is also ca. 40 residues shorter than standard [Ni,Fe]-hydrogenases essentially due to a truncated loop between helices 90:114 and 123:143 (Fig. S5).²⁴

The structure and position of the [NiFe]-center in the FrhABG structure is virtually identical to that of group 1 family members (Fig. 2b). Ni is coordinated by four cysteine thiolates (Cysa63, Cysa66, Cysa380 and Cysa383); the two latter form a bridge to Fe. Fe is further coordinated to three bi-atomic ligands modeled as two CN⁻ and one CO molecules according to infrared spectroscopic and structural data of other [NiFe]-hydrogenases.^{25; 26; 27} The two CN⁻ ligands interact with Thrα351-N and -Ogl and Argα328-N and -Nh1, respectively; both residues are conserved. The CO ligand is 3.3 Å apart from Aspα331-Od1, but cannot form a hydrogen bond (in the protonated state) for geometrical reasons. Instead, Aspα331 interacts with Hisα70 and Hisα333 and is therefore presumably charged (Fig. 2c). Aspα331 is mostly exchanged by a leucine and an asparagine in group 1 and 5 [NiFe]-hydrogenases, respectively.¹ Notably, residues of the N-terminal side of helix 67:82 of FrhA, adjacent to the [NiFe]-center, substantially differs between group 1 and 3 enzymes. Proα69 in FrhABG contacting the CO ligand is frequently exchanged by a threonine but also by a cysteine or a valine (Fig. 2c). Like group 1 enzymes FrhABG binds an additional metal ion at the protein surface 12 Å away from the [NiFe]-center. The metal ion octahedrally ligated by Gluα44-Oe2, Hisα386-Ne2, Alaα347-O and three solvent molecules was modeled as Mg²⁺. FrhA like other [NiFe]-hydrogenases is post-translationally cleaved after Hisα386 most likely by the endopeptidase FrhD.

FrhG: FrhG (40-275) begins with residue 40 mainly because we numbered according to the currently erroneous annotation of the start codon in the genome. It consists of an N-terminal flavodoxin-like domain (40-189) and a C-terminal ferredoxin domain (203-275) connected by the 18

Å long helix 190:202 (Fig. 3a). The flavodoxin-like domain composed of a five-stranded parallel β -sheet and several surrounding short helices is conserved between FrhG and group 1 enzymes reflected in a rmsd of 2.2–2.3 Å to the most related *E. coli* and *Desulfovibrio desulfuricans* species (Fig. 3a).^{7;}
²⁸The embedded proximal [4Fe-4S] cluster is ligated to Asp⁹⁶⁰ and three cysteines compared to four cysteines in group 1 family members (Fig. 3b). Due to the cysteine to aspartate exchange the invariant Glu¹⁰² is turned away but remains hydrogen-bonded to Tyr⁹⁵¹ and His⁹⁵³ both being substantially displaced (Fig. 3c). Notably, Gly⁹⁵⁶ in vander Waals contact to the [4Fe-4S] cluster in FrhG is mostly exchanged by a glutamate in group 1 members (but not in [NiFeSe]-hydrogenase). 4.4 Å apart from the proximal [4Fe-4S] cluster sits an additional cysteine (Cys¹³⁷) (Fig. 3b) which is also found in O₂-tolerant membrane-bound [NiFe]-hydrogenases.^{5; 6; 7}

In contrast to group 1 [NiFe]-hydrogenases FrhG contains a C-terminal ferredoxin domain that hosts the medial and distal [4Fe-4S] clusters (Fig. 3b, Fig. S6). The rmsd to the most related ferredoxin from *Peptostreptococcus asaccharolyticus*²⁹ is 1.7 Å using 87% of the C _{α} atoms. Both [4Fe-4S] clusters are coordinated by four cysteine sulfurs inside a hydrophobic pocket (Fig. S6). Notably, the orientation between the proximal [4Fe-4S] cluster and medial/distal Fe/S clusters are substantially different in FrhABG compared to group 1 enzymes (Fig. 3a).

At the interface of two contacting FrhABG protomers extra electron density is visible which was tentatively interpreted as two semi-occupied Zn²⁺ ions. Each of them is coordinated with two cysteine sulfurs (Cys²⁰⁶ and Cys²⁰⁸) from two different FrhABG protomers of the dimer, one putative bridging sulfur and presumably one further ligand unidentifiable (Fig. S7). The Zn²⁺ ions are ca. 3 Å apart from each other and ca. 10 Å apart from their medial [4Fe-4S] clusters.

FrhB: FrhB is built up of an eight-stranded mixed β -sheet flanked by five helices, which were topologically arranged to a novel fold (Fig. 4) already deducible to a great extent from the cryo-EM map.¹⁸ FrhB is a member of a F₄₂₀-binding protein family, which includes subunits of F₄₂₀-dependent sulfite reductase, phenazine oxidoreductase and formate dehydrogenase.⁸ The core region is composed of four parallel strands (43:48, 97:101, 127:133, 204:208) flanked by helices 28:38 and 103:114 as well as two insertion regions (Fig. 4a). While the first insertion segment primarily includes a helix (65:71), loop, helix (84:90) motif that wraps around the C-terminal side of the parallel β -

strands, the second insertion segment consists of a β -meander subdomain (137-195) which caps the central β -sheet. In addition, a C-terminal 37 Å long helix 251:275 characterized by many lysines (Lys β 256, β 261, β 262, β 264, β 267 and β 273) and leucines (Leu β 251, β 253, β 254 and β 257) is spanned over the residual FrhB perpendicular to the strands of the central β -sheet. The C-terminal end of the four central parallel β -strands forms the bottom of a narrow canyon-shaped pocket which hosts FAD (Fig. 4a+b). The pocket is accessible from the surface of the FrhABG complex and predominantly lined by aromatic and non-polar residues. The [4Fe-4S] cluster is located at the interface between FrhB and FrhG (Fig. 1a + 4c).

The conformation and binding mode of FAD in FrhABG and in most other FAD-binding protein families are clearly distinct.³⁰ FAD binds in a compact U-shaped conformation resulting in short distances between the adenine-C⁶ and the isoalloxazine dimethylbenzyl-C^{8M} of 3.5 Å and between the ribose-O⁴ and the ribitol-O^{3'} of 4.2 Å (Fig. 4b). A similar conformation of FAD was also found in photolyases and in some members of the ferredoxin-NADP⁺ reductase family.^{31; 32} The isoalloxazine ring of FAD is slightly bent and the hydride transferring N⁵ atom points towards the *Si*-face presumably caused by binding of an unknown organic species. This compound appears to be covalently linked to N⁵ and C^{4x} and forms the same five-membered ring as that found in a histone demethylase (pdb-code: 4GUU) albeit differently oriented. A physiological relevance of this modification in FrhABG is unlikely as it prevents a productive binding of F₄₂₀ (see below).

The isoalloxazine ring sits with its flat *Re*-face onto the loop after strand 129:133 (Fig. 4b) and is flanked by the loop following strand 205:208 and the loop of the helix-loop-helix segment at its xylene and pyrimidine rings, respectively, the latter being hydrogen-bonded with Met β 135-N, Tyr β 198-Oh, Ser β 209-N, -Og, and Val β 210-N. While the *Re*-face of FAD is shielded by Ile β 102, Tyr β 133 and Cys β 134, the *Si*-face is largely accessible from bulk solvent and therefore proposed as the F₄₂₀-binding site (Fig. S8). The pyrophosphate moiety is firmly anchored to the protein matrix by six amide-nitrogen atoms (Gln β 24, Asp β 25, Gly β 27, Ile β 28, Val β 29 and Thr β 30) protruding from a glycine-rich loop at the N-terminal side of helix 29:38 (Fig. 4b). The adenosine part is sandwiched between the C-terminal side of the central strands 43:48 and 97:101 and capped by the loop of the helix-loop-helix segment.

The [4Fe-4S] cluster coordinated by four cysteines (Cys β 104, Cys β 134, Cys β 192 und Cys β 195) (Fig.4c) is embedded into a positively charged surrounding, which should stabilize its reduced state. Lys β 261-Nh fixed via a hydrogen bond with Cys β 134-O and Asp β 197-O is in van der Waals contact to sulfur S1 (3.3 Å) and to iron Fe2 (3.3 Å) of the [4Fe-4S]cluster. Cys β 104N at the positively charged N-terminal side of helix 103:114 also interacts with S1. Arg γ 261-Nh1 of FrhG points to the other side of the [4Fe-4S] cluster and contactssulfur S2 (Fig. 4c).

Quaternary structure:In the crystal structure the 12 FrhABG protomersassemble to a tetraicosaedric quaternary structure with a 23 symmetry (Fig. 1) which was already deduced from recently performed cryo-EM studies.¹⁸The 1.25 MDa protein complex has a size of ca. 180 Å³ and adopts the shape of a rounded cube with an extended internal cavityfilled with bulk solvent.

The FrhABG cube is composed of six faces; each of them is built up of two FrhABG protomers that are oriented in a head-to-tail arrangement relative to each other with a two-foldaxis in the center (Fig. S7). The extended dimer interface is formed by all subunits, in particular, by the flavodoxin-like and ferredoxin domains of two FrhGsubunits andthereby buries an area ofca. 2600 Å², 4.5% of the protomer surface (Table S2). The usage of a dimer as basic unit for dodecamer formation is reminiscent tododecameric aminopeptidase,³³ cyclomaltodextrinase,³⁴ and aspartate decarboxylase.³⁵The six dimer blocks with a size of 100 x 100 x 50 Å³ are arranged in a manner that at four vertices of the cube three FrhA and at the other four vertices three FrhB coincide at the three-fold axes (Fig. 1b+c). Three insertion domains of FrhA tightly pack together therebyburying a contact area of ca. 2000Å² or 13% of the FrhA surface.Anarrowporewith a diameter of 3 Åis created at the threefold axis,which is only penetrable for small molecules like water (Fig. 1b). Three side chains of Lys α 272 points towards the threefold axis and interacts with a water moleculeplaced adjacentto the narrowest point of the pore. In contrast, the three FrhB only slightly contact each other and line up a triangular pore around the three-fold axis (Fig. 1c). This wide pore with a diameter of ca. 8 Å also allows larger molecules to enter the cavity of the FrhABG dodecamer. The inner surface of the hollow cubeis primarily linedby polar/charged residues of FrhA and FrhB whereas the outer surface is

constituted by FrhG and parts of FrhB. Based on the analyzed contact areas between the subunits an assembly process for the FrhABG complex is proposed (Fig. S9).

A rather stable dodecameric FrhABG complex¹¹ appears to be present in all methanogenic archaea independent of their thermophilic or mesophilic origin^{36; 37; 38; 39} suggesting a specific purpose for its enormous size and voluminous cavity. For comparison, other cage-like protein complexes have been assigned specific functions.⁴⁰ Sulfur oxygenase/reductase from *Acidianus ambivalens* disproportionates elementary sulfur and the pores of 8.5 Å in diameter might serve as selective filters for polysulfide that can only reach the active site from the cavity.⁴¹ Likewise, dodecameric aminopeptidases and proteasomes restrict substrate access via narrow channels such that only unfolded proteins and small peptides, respectively, can enter into the cavity and from there to the active site.^{33; 42} In the dodecameric aspartate decarboxylase the reaction velocity is controlled by cooperative substrate binding requiring a multi-subunit complex.³⁵ Ferritin stores iron in its internal cavity,^{43; 44} virus capsids envelop DNA or RNA⁴⁵ and chaperonins promote folding of unfolded proteins in a protected surrounding.⁴⁶

In the FrhABG dodecamer F₄₂₀, H₂ and protons enter/release their binding site from the outer surface of the cube (see below) implicating no apparent functional role for the cavity. A possible cooperative effect between the subunits for substrate binding was not indicated by the kinetic data. In addition, the usage of the cavity as storage media i.e. for H₂ can be nearly excluded because of the polar nature of the inner protein surface and the wide pores. Oligomerization is an established tool for increasing protein stability⁴⁷ but probably not the only reason for dodecamer formation in FrhABG. The extended inter-subunit interfaces are described in Table S2. A plausible function would be the protection of the redox centers in FrhABG against undesirable redox-active compounds/proteins because of their higher concentrations in the cytosol compared to the periplasm and the low redox potential of the [4Fe-4S] clusters. FrhABG is localized in the cytoplasm and the group 1 [NiFe]-hydrogenases in the periplasm. The structure partly supports this idea as the two [4Fe-4S] clusters of the ferredoxin module of FrhG are ca. 7 Å apart from bulk solvent in the FrhABG protomer and 8-12 Å in the FrhABG dodecamer.

Structural basis of the catalytic reaction

The H₂ oxidation site: The structure of the [NiFe]-center and presumably also the H₂ activation mechanism of FrhABG is very similar to that of group 1 [NiFe]-hydrogenases.⁴⁸ Significant differences in the first interaction shell between polypeptide and [NiFe]-center were only observed at the back-stop position of the CO ligand of Fe caused by a leucine to aspartate exchange (Fig. 2b).

Dependent on the redox and substrate binding state the structure of the [NiFe]-center is subjected to small modifications. Crystal structures of group 1 [NiFe]-hydrogenases were characterized in the Ni-EPR detectable, oxidized and inactive Ni-A and Ni-B, and in the Ni-EPR-silent, reduced and active forms.⁴⁸ In the Ni-A form, one of the terminal cysteine-sulfur ligands (Cys380 in FrhA) of Ni and one of the cysteine sulfur ligands (Cys66 in FrhA) bridging Fe and Ni were modified^{26; 27} presumably by oxidation with an oxygen.⁴⁹ In the Ni-B form of the *Desulfovibrio vulgaris* Miyazaki F enzyme only the terminal sulfur ligand (Cys380 in FrhA) was largely oxidized with oxygen²⁷ while in the *D. fructosovorans* enzyme no sulfur was oxidized.²⁶ In the Ni-A and Ni-B forms Ni and Fe are bridged by peroxide and hydroxyl ligands^{26; 27} but in the active reduced state no bridging ligand is visible in group 1 enzymes⁵⁰ (albeit a bridging hydride ligand is found by EPR methods⁵¹). The “as isolated” FrhABG complex reveals no Ni-EPR signal suggesting a Ni²⁺ oxidation state and no bridging ligand between Ni and Fe (Fig. 2b). A peak in the $F_o - F_c$ electron density map (3.5 Å), asymmetrically arranged between the Ni and Fe, could not be refined and is considered as crystallographic artifact. The distances between Ni and Fe are 2.8–2.9 Å and 2.7–2.8 Å in the Ni-A and Ni-B forms, respectively,^{26; 27} and 2.55 Å in reduced forms of group 1 enzymes^{48; 50} while 2.75 Å in the “as isolated” FrhABG complex. Although not definitively worked out, the data rather argues for a reduced [NiFe]-center.

H₂ transfer: It is proposed that H₂ (also O₂ and CO) preferentially reaches the buried [NiFe]-center from the solvent-accessible surface via a network of hydrophobic channels; several routes were discerned from previous structural data and frequently conserved in FrhA and FrhG including the putative principal gas channel.^{48; 52} FrhA, a truncated version of the large subunit of group 1 family members, contains a shorter linker segment between helices 90:114 and 123:143 of the four-helix bundle domain which offers one channel to be dominantly used for H₂ transport (Fig. 5a; Fig. S10). It

is located at the interface of FrhA and FrhG, and is with a length of ca. 20 Å shorter than those traced in group 1 enzymes. Leu γ 75, Ile γ 81, Leu γ 87 and Phe α 111 constitute a pore from the outer surface of the FrhABG dodecamer to the conserved hydrophobic channel network in the protein interior. From the pore the channel extends along Leu γ 64, Thr γ 65, Leu γ 87, Met γ 62, Ile α 108, Leu α 112, Val α 65, Arg α 328 and Glu α 14 to the Ni atom (Fig. 5a). No solvent molecule is visible along this pathway.

Proton transfer: An analysis of the FrhABG structure revealed one preferential proton relay route which is present in several variants and related to that found in other [NiFe]-hydrogenases.⁵³ It starts at the conserved Glu α 14 positioned in van der Waals contact to the Ni ligating Cys α 380. Its carboxylate side chain maintains its conformation but interacts not only with Thr α 58-Og1, Leu α 381-N and Ser α 382-N but also with Ser α 382-Og and His γ 53-Ne2 (Fig. 5b). The latter two residues are replaced in group 1 enzymes by an alanine and spatially by glutamate, respectively. Notably, the imidazole side chain of the invariant His γ 53 has moved ca. 4 Å and forms a salt bridge to Glu γ 102 which has also altered its position due to the exchange of acysteine by an aspartate as a ligand of the proximal [4Fe-4S] cluster (Fig. 3c). One variant of the proton transfer pathway proceeds from Glu α 14 via His γ 53 and six firmly bound water molecules to the bulk solvent (Fig. 5b). His γ 53-Ne2, Thr α 38-Og, Leu γ 54-O, Ser γ 112-Og, His α 16-Ne2, His γ 111-Ne2 and Ser α 8-Og link these water molecules to the protein matrix. A second proton transfer route proceeds from Glu α 14-Oe2, Thr γ 58-Og1 and several water molecules to the bulk solvent. The water molecules are fixed by Gly α 15-N, Gln γ 85-N, -Oe1, Thr γ 86-Og1, Tyr γ 51-O, His γ 53-N, His α 16-Ne2, Glu γ 115-Oe2, His γ 111-Ne2 and Ser α 8-Og. The protons of the described and related pathways are released into bulk solvent close to the FAD binding pocket of FrhB of a neighboring dimer at the outer surface of the FrhABG dodecamer. Theoretical studies also favor the proton channel starting from Glu α 14.^{54; 55} The proton transfer routes and the involved amino acid residues are only approximately conserved in [Ni,Fe] hydrogenases; differences between FrhABG and the *D. fructosovorans* enzyme are illustrated in Fig. S11. An alternative proton transfer route that passes the putative Mg²⁺ ion is postulated for most [NiFe]-hydrogenases but is less likely for FrhABG as no continuous chain of proton donors/acceptors is formed.

F₄₂₀-binding and hydride transfer: The F₄₂₀-binding site is postulated to be located in FrhB next to the isoalloxazine ring of FAD (Fig. 4b) which was confirmed by the found F₄₂₀H₂ oxidizing activity of heterologously produced FrhB (Fig. S3). Although a crystal structure of the FrhABG-F₄₂₀ complex could not be determined, it is possible to reliably model F₄₂₀ into FrhB on the basis of the following restraints: (1) N⁵ of FAD and C⁵ of F₄₂₀ have to be in van-der-Waals contact to each other for an hydride transfer; (2) F₄₂₀ stereospecifically transfers the hydride from the *Si*-face of the deazaisoalloxazine ring as found for all F₄₂₀ dependent enzymes;⁵⁶ and (3) F₄₂₀ has to be placed into its binding site in a manner which minimizes its collisions with the polypeptide scaffold and FAD (Fig. S8).

Accordingly, the deazaisoalloxazine ring of F₄₂₀ is embedded between the *Si*-face of FAD and the β -meander subdomain into a solvent-accessible pocket. The long ribitol, phosphate and polyglutamate tail is directed to the opening of the pocket flanked by the β -meander and the C-terminal helix of FrhB. Tyr β 76 and Glu β 136 collide with the modeled deazaisoalloxazine ring. A conformational change of their side chains upon F₄₂₀ binding is therefore necessary (Fig. S8). Evading F₄₂₀, the phenol side chain of Tyr β 76 might rotate 90° to stack to the *Re*-side of the tricyclic ring and the carboxylate group of Glu β 136 might swing towards the ribitol group of F₄₂₀. As mentioned, F₄₂₀ binding would interfere with the found modification at FAD. While the *Si*-side of F₄₂₀ faces the *Si*-side of FAD, the *Re*-side is shielded by Leu β 181, Ile β 163 and presumably Tyr β 76.

The electron flow between the two active sites: H₂ oxidation and F₄₂₀ reduction, ca. 45 Å spatially separated in one FrhABG protomer, are coupled by an exchange of two electrons shuttled – one by one – via four [4Fe-4S] clusters between the [NiFe]-center and FAD (Fig. 1a).¹⁸ Adjacent electron carriers in one FrhABG protomer are spaced sufficiently close to ensure physiological relevant electron transfer rates between the two active sites.^{3; 57; 58; 59} The edge-to-edge distances between the [NiFe]-center (Cys α 63-Sg) and the proximal [4Fe-4S] cluster (Cys γ 57-Sg) are 6.2 Å, between the proximal [4Fe-4S] cluster (Cys γ 171-Sg) and the medial [4Fe-4S] cluster (Cys γ 252-Sg) 6.2 Å, between the medial [4Fe-4S] cluster (Cys γ 255-Sg) and the distal [4Fe-4S] cluster (Cys γ 226-Sg) 6.6 Å, between the distal [4Fe-4S] cluster (Cys γ 223-Sg) and the [4Fe-4S] cluster of FrhB (Cys β 104-Sg) 6.1 Å and between the latter [4Fe-4S] cluster (Cys β 134-Sg) and FAD (C6) 3.5 Å. Two redox centers are either in

contact to each other via two ligating cysteines or one bridging residue between them (Fig. 1a). Electron flow between different FrhABG protomers of the dodecameric complex is unlikely although the distance of 17 Å between the medial [4Fe-4S] clusters of two FrhABG protomers with an intermediate putative dimetallic cluster on the dimer axis does not completely exclude this option (Fig. S7). Interestingly, the electron transfer pathways of FrhABG and group 1 [NiFe]-hydrogenases are only conserved between the [NiFe]-center and the proximal [4Fe-4S] cluster which is consistent with the participation of the latter in H₂ activation. In contrast, the medial and distal [4Fe-4S] clusters are only applied for electron conduction. The ferredoxin domain of FrhG is an adequate module for this purpose (Figs. 3a + S6).

The FrhABG reaction is used in methanogenic archaea in the forward and backward directions. *In vitro* studies, indeed, indicate the oxidation and production of H₂ with the same rates.¹² Equilibrium between both directions is accomplished by similar electrochemical potentials of the H₂/H⁺, F₄₂₀H₂/F₄₂₀ and [4Fe-4S]⁺/[4Fe-4S]²⁺ redox pairs if the distances between the electron carriers are sufficiently short as found in FrhABG. EPR spectroscopic measurements resulted in redox potentials of the intervening [4Fe-4S] clusters of around -400 mV (Fig. S4), which approximately corresponds to that measured for the F₄₂₀-reducing [NiFeSe]-hydrogenase from *M. voltae* (-370 mV).²² The redox potential of the [NiFe]-center is assumed to be similar to that of the H₂/H⁺ couple (E' = -414 mV) while the physiological redox potentials of F₄₂₀ is -350 mV, which was determined under 0.3 to 35% H₂ in argon atmosphere in the presence of 20 µM deazaflavin.⁶⁰ The standard redox potential of free FAD in solution is -205 mV but its redox potential can change by the polypeptide surrounding by more than 200 mV.⁶¹ In FrhABG, FAD is surrounded by four hydrogen donors, no positively charged residue or helix dipoles but two aspartates, which might induce a more negative redox potential. Thus, the electrochemical potential of all redox processes appears to be on the required similar level between ca. -300 and -400 mV.

[NiFe]-hydrogenases, studied so far, predominantly catalyze *in vivo* the H₂ oxidation reaction. The most obvious differences between them and FrhABG are the higher redox potential of the electron accepting cytochrome compared to F₄₂₀ and the H₂/H⁺ pair and the discrepancies among the redox potentials of the intervening Fe/S clusters (Table 1). The type of the medial iron-sulfur cluster appears

to play a crucial role which is in FrhABG a [4Fe-4S] cluster and in most group 1 enzymes a [3Fe-4S] cluster the latter characterized by a significantly higher redox potential. The conversion of the medial [3Fe-4S] cluster to a [4Fe-4S] cluster in [NiFe]-hydrogenase of *D. fructosovorans* by site-specific amino acid exchange reduces the redox potential by ca. 300 mV, and significantly decreases the H₂ oxidation and increases the proton reduction reaction.⁶² The reverse conversion of the medial [4Fe-4S] to a [3Fe-4S] cluster in FrhABG of *M. voltae* raises the redox potential by about 400 mV and decreases the reduction rate of F₄₂₀ ten-fold²² suggesting that the electron transfer between the medial and distal cluster become slower and rate-limiting for the FrhABG reaction. [NiFeSe]-hydrogenases, which contain a medial [4Fe-4S] cluster^{24; 63} like FrhG and not a [3Fe-4S] cluster catalyze both directions with comparable rates.⁶⁴ The redox potential and the ratio of the forward and backward reactions are also determined by the direct protein ligands of the iron-sulfur clusters as demonstrated by the mutation of a histidine to a cysteine or glycine ligand at the distal [4Fe-4S] cluster of [Ni,Fe]-hydrogenase from *D. fructosovorans* (Table 1). Ligand exchange reduces the electron transfer rates significantly more in the H₂ oxidation than in the proton reduction reaction.⁶⁵ In addition, the exchange of a cysteine by an aspartate as a ligand of the [4Fe-4S] cluster in ferredoxin was shown to affect its redox potential and electron transfer properties^{66; 67} which might also be used by FrhABG which contains an aspartate ligated proximal [4Fe-4S] cluster.

Materials and methods

Cultivation of *M. marburgensis*

M. marburgensis (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen (DSMZ). The archaeon was anaerobically grown at 65 °C on 80% H₂/20% CO₂/0.1% H₂S in a 12 L fermenter containing 10 L complete mineral salt medium.⁶⁸ When the cells reached the late exponential phase the fermenter was cooled down to 4 °C. Cells were harvested using a continuous-flow centrifuge under anoxic conditions and stored at -80 °C.

Purification of the Frh complex from *M. marburgensis*

Purification was performed under strictly anaerobic conditions at 18 °C in an anaerobic chamber (Coy Laboratory Products) as described previously.¹⁸ All anaerobic buffers used contained 2 mM DTT and 25 µM FAD.

Heterologous expression of *frhB*

The *frhB* gene was amplified by PCR with high-fidelity Phusion DNA polymerase (New England Biolabs GmbH, Frankfurt, Germany) using the *Methanocaldococcus jannaschii* genomic DNA as a template. The gene was additionally tagged with a 3' His₆ cassette. The applied primers were: 5'-AGCCATATGATGAGGTGGAATAGGATGAATCCTTTTGGTAGC-3' (forward primer; *NdeI* restriction side is underlined) and 5'-CAGTCTCGAGGTATGGAATAGGTAGTCCGAGCTCTTTTCAATG-3' (reverse primer; *XhoI* restriction side is underlined). The blunt PCR product was ligated into the pCR Blunt vector (Invitrogen, Carlsbad, California, USA), which was subsequently introduced into the *E. coli* TOP10 strain. The *NdeI/XhoI* fragment was cloned into the expression vector pET24b(+) by using *E. coli* TOP10; the cloned fragment was sequenced. The resulting vector containing *frhB* was introduced again into *E. coli* BL21(DE3) for expression. The cells were aerobically grown in 2L of LB medium at 37°C with stirring at 550 rpm. When an optical density at 600 nm had reached 0.8–1.0, the gene expression of *frhB* was started by adding isopropyl β-d-thiogalactopyranoside (0.5 mM, final concentration) as well as cysteine (0.12 g/L), ferrous sulfate (0.1 g/L), ferric citrate (0.1 g/L), and ferric ammonium citrate (0.1 g/L) to promote [4Fe-4S] cluster formation. After 20 h cultivation at room temperature under stirring at 250 rpm, the cells were harvested by centrifugation and stored anaerobically at -80°C.

Purification of FrhB

Purification of His-tagged FrhB was performed under strictly anoxic conditions at 18°C in an anaerobic chamber. All buffers used contained 2 mM DTT and 10 µM FAD. 5 g (wet mass) of *E. coli* cells were resuspended in a solution of 15 mL containing 20 mM imidazole, 150 mM NaCl, 50 mM Tris/HCl pH 7.5, and passed four times through a French pressure cell at 125 MPa. Cell debris and

membrane were removed by centrifugation at $150,000 \times g$ and $4\text{ }^{\circ}\text{C}$ for 30 min. The supernatant contained ~120 mg protein and was subjected to purification described in Fig. S2. The iron content of the enzyme was determined colorimetrically with ferene (3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazinedisodium trihydrate). Mohr's salt was used as the standard according to a procedure described previously.⁶⁹

Enzyme assay

The enzyme assays were performed in anaerobic 1.5 mL quartz cuvettes, which were sealed with butyl rubber stoppers. All components were added anoxically by Hamilton syringes. The reduction of F_{420} to $F_{420}H_2$ by FrhABG was performed at $70\text{ }^{\circ}\text{C}$. The anaerobic 0.7 mL mixture contained 100 mM HEPES/HCl, pH 7.5, $40\text{ }\mu\text{M}$ F_{420} ($\epsilon_{401} = 25.6\text{ mM}^{-1}\text{ cm}^{-1}$ at pH 7.5) and $10\text{ }\mu\text{M}$ sodium dithionite. Volume of the gas phase was 0.2 mL, which was filled with 100% H_2 . The reaction was started by addition of FrhABG ($1\text{--}10\text{ }\mu\text{g}$) and followed by the decrease of the absorbance at 401 nm.

Crystallization

Crystallization was performed at $8\text{ }^{\circ}\text{C}$ under strictly anoxic conditions in an anaerobic chamber by the sitting-drop vapor diffusion method using 24-well plates (Jena Bioscience GmbH, Jena). The best crystals were obtained when mixing $1\text{ }\mu\text{L}$ of enzyme solution containing 15 mg mL^{-1} , 10 mM MES/NaOH, pH 6.0, $25\text{ }\mu\text{M}$ FAD, and 2 mM DTT with $1\text{ }\mu\text{L}$ of reservoir solution. The reservoir solution is composed of 100 mM MES/NaOH, pH 6.5, 17–20 % PEG797, 400 mM $MgCl_2$, 5% glycerol, and 100 mM trimethylamineoxide. Dark-brown crystal grew to a size of $0.4 \times 0.4 \times 0.4\text{ mm}^3$ within 2–3 weeks (Fig. S1) and could be directly flash-frozen in a liquid nitrogen stream.

Collection of X-ray diffraction data, processing, and structure determination

Native and anomalous data were collected at the beamline PXII at the Swiss-Light-Source (SLS) in Villigen (Switzerland) and processed with XDS⁷⁰ (Table S1). Initial phases were calculated by the SAD method (SHARP⁷¹) at $2.5\text{ }\text{\AA}$ resolution using a “peak” data set measured at the iron edge. The positions of the irons of the $[4Fe-4S]$ clusters were first detected with SHELXD.⁷² The quality of

the solvent-flattened map (SOLOMON⁷³) was not sufficient for model building. Therefore, the phases calculated from the cryo-EM density of the FrhABG complex¹⁸ and the calculated SAD phases were combined and solvent-flattened. Now, the protein model could be built using COOT.⁷⁴ Homologous structures of [NiFe]-hydrogenase of *A. vinosum* (PDB-Code: 3myr, sequence identity: 28%), ferredoxin from *P. asaccharolyticus* (PDB-Code: 1dur, sequence identity: 32%) and the partial model from the cryo-EM map were used for interpretation of the polypeptide backbone. The obtained first FrhABG model was improved with PHENIX⁷⁵ and subsequently refined with REFMAC5.⁷⁶ Its final geometrical quality was assessed with COOT and PHENIX. Figures were prepared with PyMOL (Schrödinger, LLC).

Determination of the redox potential

Redox potentials were EPR spectroscopically determined from samples obtained after potentiometric titrations, similar to Wilson and Dutton.^{77; 78} The titration experiment under anaerobic conditions contained 200 μ M FrhB or 40 μ M FrhABG, respectively. The titration was carried out by adjusting the redox potential with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) or potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$). Following redox mediators were used at a final concentration of 100 μ M: menadione (-13 mV), 2-hydroxy-1,4-naphthoquinone (-152 mV), anthraquinone-2-sulfonate (-225 mV), safranin T (-289 mV), benzylviologen (-358 mV), and methylviologen (-449 mV). The redox potentials were measured on an Ingold Pt 4805-S7/120 Pt/Ag/AgCl electrode and are quoted relative to the normal standard hydrogen electrode. After adjusting the desired potential, 270 μ L samples were anaerobically loaded into calibrated quartz EPR tubes and rapidly frozen in liquid nitrogen. Spectra were recorded with a Bruker EMX-6/1 X band EPR spectrometer equipped with an Oxford Instruments helium flow cryostat.

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The crystal structure of FrhABG without F₄₂₀ has been deposited in the Protein Data Bank with accession PDB code 4OMF.

Literature

1. Vignais, P. M. & Billoud, B. (2007). Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev***107**, 4206-72.
2. Constant, P., Chowdhury, S. P., Pratscher, J. & Conrad, R. (2010). *Streptomyces* contributing to atmospheric molecular hydrogen soil uptake are widespread and encode a putative high-affinity [NiFe]-hydrogenase. *Environ Microbiol***12**, 821-9.
3. Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M. & Fontecilla-Camps, J. C. (1995). Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature***373**, 580-7.
4. Massanz, C., Schmidt, S. & Friedrich, B. (1998). Subforms and in vitro reconstitution of the NAD-reducing hydrogenase of *Alcaligenes eutrophus*. *J Bacteriol***180**, 1023-9.
5. Fritsch, J., Scheerer, P., Frielingsdorf, S., Kroschinsky, S., Friedrich, B., Lenz, O. & Spahn, C. M. (2011). The crystal structure of an oxygen-tolerant hydrogenase uncovers a novel iron-sulphur centre. *Nature***479**, 249-52.
6. Shomura, Y., Yoon, K. S., Nishihara, H. & Higuchi, Y. (2011). Structural basis for a [4Fe-3S] cluster in the oxygen-tolerant membrane-bound [NiFe]-hydrogenase. *Nature***479**, 253-6.
7. Volbeda, A., Amara, P., Darnault, C., Mouesca, J. M., Parkin, A., Roessler, M. M., Armstrong, F. A. & Fontecilla-Camps, J. C. (2012). X-ray crystallographic and computational studies of the O₂-tolerant [NiFe]-hydrogenase 1 from *Escherichia coli*. *Proc Natl Acad Sci U S A***109**, 5305-10.
8. Thauer, R. K., Kaster, A. K., Goenrich, M., Schick, M., Hiromoto, T. & Shima, S. (2010). Hydrogenases from methanogenic archaea, nickel, a novel cofactor, and H₂ storage. *Annu Rev Biochem***79**, 507-36.
9. Jacobson, F. S., Daniels, L., Fox, J. A., Walsh, C. T. & Orme-Johnson, W. H. (1982). Purification and properties of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*. *J Biol Chem***257**, 3385-8.
10. Eirich, L. D., Vogels, G. D. & Wolfe, R. S. (1978). Proposed structure for coenzyme F₄₂₀ from *Methanobacterium*. *Biochemistry***17**, 4583-93.
11. Fox, J. A., Livingston, D. J., Orme-Johnson, W. H. & Walsh, C. T. (1987). 8-Hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*: 1. Purification and characterization. *Biochemistry***26**, 4219-27.
12. Livingston, D. J., Fox, J. A., Orme-Johnson, W. H. & Walsh, C. T. (1987). 8-Hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*: 2. Kinetic and hydrogen-transfer studies. *Biochemistry***26**, 4228-37.
13. Kulkarni, G., Kridelbaugh, D. M., Guss, A. M. & Metcalf, W. W. (2009). Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*. *Proc Natl Acad Sci U S A***106**, 15915-20.
14. Kim, Y. J., Lee, H. S., Kim, E. S., Bae, S. S., Lim, J. K., Matsumi, R., Lebedinsky, A. V., Sokolova, T. G., Kozhevnikova, D. A., Cha, S. S., Kim, S. J., Kwon, K. K., Imanaka, T., Atomi, H., Bonch-Osmolovskaya, E. A., Lee, J. H. & Kang, S. G. (2010). Formate-driven growth coupled with H₂ production. *Nature***467**, 352-5.
15. Albracht, S. P. (1994). Nickel hydrogenases: in search of the active site. *Biochim Biophys Acta***1188**, 167-204.

16. Alex, L. A., Reeve, J. N., Orme-Johnson, W. H. & Walsh, C. T. (1990). Cloning, sequence determination, and expression of the genes encoding the subunits of the nickel-containing 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum* delta H. *Biochemistry***29**, 7237-44.
17. Theodoratou, E., Huber, R. & Bock, A. (2005). [NiFe]-hydrogenase maturation endopeptidase: structure and function. *Biochem Soc Trans***33**, 108-11.
18. Mills, D. J., Vitt, S., Strauss, M., Shima, S. & Vonck, J. (2013). De novo modeling of the F₄₂₀-reducing [NiFe]-hydrogenase from a methanogenic archaeon by cryo-electron microscopy. *Elife***2**, e00218.
19. Allegretti, M., Mills, D. J., McMullan, G., Kuhlbrandt, W. & Vonck, J. (2014). Atomic model of the F₄₂₀-reducing [NiFe] hydrogenase by electron cryo-microscopy using a direct electron detector. *Elife***3**, e01963.
20. Frey, M. (2002). Hydrogenases: hydrogen-activating enzymes. *Chembiochem***3**, 153-60.
21. Hagemeyer, C. H., Shima, S., Thauer, R. K., Bourenkov, G., Bartunik, H. D. & Ermler, U. (2003). Coenzyme F₄₂₀-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) from *Methanopyrus kandleri*: a methanogenic enzyme with an unusual quarternary structure. *J Mol Biol***332**, 1047-57.
22. Bingemann, R. & Klein, A. (2000). Conversion of the central [4Fe-4S] cluster into a [3Fe-4S] cluster leads to reduced hydrogen-uptake activity of the F₄₂₀-reducing hydrogenase of *Methanococcus voltae*. *Eur J Biochem***267**, 6612-8.
23. Higuchi, Y., Yagi, T. & Yasuoka, N. (1997). Unusual ligand structure in Ni-Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis. *Structure***5**, 1671-80.
24. Marques, M. C., Coelho, R., De Lacey, A. L., Pereira, I. A. & Matias, P. M. (2010). The three-dimensional structure of [NiFeSe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough: a hydrogenase without a bridging ligand in the active site in its oxidised, "as-isolated" state. *J Mol Biol***396**, 893-907.
25. Happe, R. P., Roseboom, W., Pierik, A. J., Albracht, S. P. & Bagley, K. A. (1997). Biological activation of hydrogen. *Nature***385**, 126.
26. Volbeda, A., Martin, L., Cavazza, C., Matho, M., Faber, B. W., Roseboom, W., Albracht, S. P., Garcin, E., Rousset, M. & Fontecilla-Camps, J. C. (2005). Structural differences between the ready and unready oxidized states of [NiFe] hydrogenases. *J Biol Inorg Chem***10**, 239-49.
27. Ogata, H., Hirota, S., Nakahara, A., Komori, H., Shibata, N., Kato, T., Kano, K. & 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**, 1635-42.
28. Matias, P. M., Soares, C. M., Saraiva, L. M., Coelho, R., Morais, J., Le Gall, J. & Carrondo, M. A. (2001). [NiFe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 27774: gene sequencing, three-dimensional structure determination and refinement at 1.8 Å and modelling studies of its interaction with the tetrahaem cytochrome *c3*. *J Biol Inorg Chem***6**, 63-81.
29. Adman, E. T., Siefker, L. C. & Jensen, L. H. (1976). Structure of *Peptococcus aerogenes* ferredoxin. Refinement at 2 Å resolution. *J Biol Chem***251**, 3801-6.
30. Dym, O. & Eisenberg, D. (2001). Sequence-structure analysis of FAD-containing proteins. *Protein Sci***10**, 1712-28.
31. Chaves, I., Pokorny, R., Byrdin, M., Hoang, N., Ritz, T., Brettel, K., Essen, L. O., van der Horst, G. T., Batschauer, A. & Ahmad, M. (2011). The cryptochromes: blue light photoreceptors in plants and animals. *Annu Rev Plant Biol***62**, 335-64.
32. Karplus, P. A. & Faber, H. R. (2004). Structural Aspects of Plant Ferredoxin : NADP(+) Oxidoreductases. *Photosynth Res***81**, 303-15.
33. Russo, S. & Baumann, U. (2004). Crystal structure of a dodecameric tetrahedral-shaped aminopeptidase. *J Biol Chem***279**, 51275-81.
34. Lee, H. S., Kim, M. S., Cho, H. S., Kim, J. I., Kim, T. J., Choi, J. H., Park, C., Lee, H. S., Oh, B. H. & Park, K. H. (2002). Cyclomaltodextrinase, neopullulanase, and maltogenic amylase are nearly indistinguishable from each other. *J Biol Chem***277**, 21891-7.
35. Chen, H. J., Ko, T. P., Lee, C. Y., Wang, N. C. & Wang, A. H. (2009). Structure, assembly, and mechanism of a PLP-dependent dodecameric L-aspartate beta-decarboxylase. *Structure***17**, 517-29.

36. Braks, I. J., Hoppert, M., Roge, S. & Mayer, F. (1994). Structural aspects and immunolocalization of the F₄₂₀-reducing and non-F₄₂₀-reducing hydrogenases from *Methanobacterium thermoautotrophicum* Marburg. *J Bacteriol***176**, 7677-87.
37. Fiebig, K. & Friedrich, B. (1989). Purification of the F₄₂₀-reducing hydrogenase from *Methanosarcina barkeri* (strain Fusaro). *Eur J Biochem***184**, 79-88.
38. Sprott, G. D., Shaw, K. M. & Beveridge, T. J. (1987). Properties of the particulate enzyme F₄₂₀-reducing hydrogenase isolated from *Methanospirillum hungatei*. *Can.J.Microbiol.***33**, 896-904.
39. Muth, E., Morschel, E. & Klein, A. (1987). Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium *Methanococcus voltae*. *Eur J Biochem***169**, 571-7.
40. Dutta, S. & Berman, H. M. (2005). Large macromolecular complexes in the Protein Data Bank: a status report. *Structure***13**, 381-8.
41. Urich, T., Gomes, C. M., Kletzin, A. & Frazao, C. (2006). X-ray Structure of a self-compartmentalizing sulfur cycle metalloenzyme. *Science***311**, 996-1000.
42. Groll, M. & Huber, R. (2003). Substrate access and processing by the 20S proteasome core particle. *Int J Biochem Cell Biol***35**, 606-16.
43. Crichton, R. R. & Declercq, J. P. (2010). X-ray structures of ferritins and related proteins. *Biochim Biophys Acta***1800**, 706-18.
44. Bou-Abdallah, F. (2010). The iron redox and hydrolysis chemistry of the ferritins. *Biochim Biophys Acta***1800**, 719-31.
45. Roos, W. H., Ivanovska, I. L., Evilevitch, A. & Wuite, G. J. (2007). Viral capsids: mechanical characteristics, genome packaging and delivery mechanisms. *Cell Mol Life Sci***64**, 1484-97.
46. Tang, Y. C., Chang, H. C., Roeben, A., Wischnewski, D., Wischnewski, N., Kerner, M. J., Hartl, F. U. & Hayer-Hartl, M. (2006). Structural features of the GroEL-GroES nano-cage required for rapid folding of encapsulated protein. *Cell***125**, 903-14.
47. Walden, H., Bell, G. S., Russell, R. J., Siebers, B., Hensel, R. & Taylor, G. L. (2001). Tiny TIM: a small, tetrameric, hyperthermostable triosephosphate isomerase. *J Mol Biol***306**, 745-57.
48. Ogata, H., Lubitz, W. & Higuchi, Y. (2009). [NiFe] hydrogenases: structural and spectroscopic studies of the reaction mechanism. *Dalton Trans*, 7577-87.
49. Carepo, M., Tierney, D. L., Brondino, C. D., Yang, T. C., Pamplona, A., Telser, J., Moura, I., Moura, J. J. & Hoffman, B. M. (2002). ¹⁷O ENDOR detection of a solvent-derived Ni-(OH_x)-Fe bridge that is lost upon activation of the hydrogenase from *Desulfovibrio gigas*. *J Am Chem Soc***124**, 281-6.
50. Higuchi, Y., Ogata, H., Miki, K., Yasuoka, N. & Yagi, T. (1999). Removal of the bridging ligand atom at 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**, 549-56.
51. Brecht, M., van Gastel, M., Buhrke, T., Friedrich, B. & 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**, 13075-83.
52. Volbeda, A., Montet, Y., Vernede, X., Hatchikian, E. C. & Fontecilla-Camps, J. C. (2002). High-resolution crystallographic analysis of *Desulfovibrio fructosovorans* [Ni,Fe] hydrogenase. *Int. J. Hyd. Energy***27**, 1449-1461.
53. Fontecilla-Camps, J. C., Volbeda, A., Cavazza, C. & Nicolet, Y. (2007). Structure/function relationships of [NiFe]- and [FeFe]-hydrogenases. *Chem Rev***107**, 4273-303.
54. Fdez Galvan, I., Volbeda, A., Fontecilla-Camps, J. C. & Field, M. J. (2008). A QM/MM study of proton transport pathways in a [NiFe] hydrogenase. *Proteins***73**, 195-203.
55. Teixeira, V. H., Soares, C. M. & Baptista, A. M. (2008). Proton pathways in a [NiFe]-hydrogenase: A theoretical study. *Proteins***70**, 1010-22.
56. Klein, A. R., Berk, H., Purwantini, E., Daniels, L. & Thauer, R. K. (1996). Si-face stereospecificity at C5 of coenzyme F₄₂₀ for F₄₂₀-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium smegmatis* and F₄₂₀-dependent alcohol dehydrogenase from *Methanoculleus thermophilicus*. *Eur J Biochem***239**, 93-7.

57. Page, C. C., Moser, C. C., Chen, X. & Dutton, P. L. (1999). Natural engineering principles of electron tunnelling in biological oxidation-reduction. *Nature***402**, 47-52.
58. Prytkova, T. R., Kurnikov, I. V. & Beratan, D. N. (2007). Coupling coherence distinguishes structure sensitivity in protein electron transfer. *Science***315**, 622-5.
59. Winkler, J. R. & Gray, H. B. (2014). Long-Range Electron Tunneling. *J Am Chem Soc.*
60. Jacobson, F. S. & Walsh, C. T. (1983). Properties of 7,8-dimethyl-8-hydroxy-5-deazaflavins relevant to redox coenzyme function in methanogen metabolism. *Biochemistry***23**, 979-988.
61. Buckel, W. & Thauer, R. K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na⁺ translocating ferredoxin oxidation. *Biochim Biophys Acta***1827**, 94-113.
62. Rousset, M., Montet, Y., Guigliarelli, B., Forget, N., Asso, M., Bertrand, P., Fontecilla-Camps, J. C. & Hatchikian, E. C. (1998). [3Fe-4S] to [4Fe-4S] cluster conversion in *Desulfovibrio fructosovorans* [NiFe] hydrogenase by site-directed mutagenesis. *Proc Natl Acad Sci U S A***95**, 11625-30.
63. Garcin, E., Vernede, X., Hatchikian, E. C., Volbeda, A., Frey, M. & Fontecilla-Camps, J. C. (1999). The crystal structure of a reduced [NiFeSe] hydrogenase provides an image of the activated catalytic center. *Structure***7**, 557-66.
64. Baltazar, C. S. A., Marques, M. C., Soares, C. M., DeLacey, A. M., Pereira, I. A. C. & Matias, P. M. (2011). Nickel–Iron–Selenium Hydrogenases – An Overview. *European Journal of Inorganic Chemistry***2011**, 948–962.
65. Dementin, S., Belle, V., Bertrand, P., Guigliarelli, B., Adryanczyk-Perrier, G., De Lacey, A. L., Fernandez, V. M., Rousset, M. & Leger, C. (2006). Changing the ligation of the distal [4Fe4S] cluster in NiFe hydrogenase impairs inter- and intramolecular electron transfers. *J Am Chem Soc***128**, 5209-18.
66. Calzolari, L., Gorst, C. M., Zhao, Z. H., Teng, Q., Adams, M. W. & La Mar, G. N. (1995). ¹H NMR investigation of the electronic and molecular structure of the four-iron cluster ferredoxin from the hyperthermophile *Pyrococcus furiosus*. Identification of Asp14 as a cluster ligand in each of the four redox states. *Biochemistry***34**, 11373-84.
67. Zhou, Z. H. & Adams, M. W. (1997). Site-directed mutations of the 4Fe-ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus*: role of the cluster-coordinating aspartate in physiological electron transfer reactions. *Biochemistry***36**, 10892-900.
68. Schönheit, P., Moll, J. & Thauer, R. K. (1980). Growth parameters (K_s, m_{max}, Y_s) of *Methanobacterium thermoautotrophicum*. *Arch Microbiol***127**, 59-65.
69. Janda, M. & Hemmerich, P. (1976). 5-Deaza- and 5-thiariboflavins: a simple pathway to antimetabolites of vitamin B₂. *Angew Chem Int Ed Engl***15**, 443-4.
70. Kabsch, W. (2010). Xds. *Acta Crystallogr D Biol Crystallogr***66**, 125-32.
71. De la Fortelle, E. & Bricogne, G. (1997). Maximum likelihood heavy-atom parameter refinement for multiple isomorphous replacement and multiwavelength anomalous diffraction methods. *Methods Enzymol***276**, 472-494.
72. Schneider, T. R. & Sheldrick, G. M. (2002). Substructure solution with SHELXD. *Acta Crystallogr D Biol Crystallogr***58**, 1772-9.
73. Abrahams, J. P. & Leslie, A. G. (1996). Methods used in the structure determination of bovine mitochondrial F1 ATPase. *Acta Crystallogr D Biol Crystallogr***52**, 30-42.
74. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr***60**, 2126-32.
75. Afonine, P. V., Grosse-Kunstleve, R. W., Chen, V. B., Headd, J. J., Moriarty, N. W., Richardson, J. S., Richardson, D. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2010). phenix.model_vs_data: a high-level tool for the calculation of crystallographic model and data statistics. *J Appl Crystallogr***43**, 669-676.
76. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr***53**, 240-55.
77. Dutton, P. L. (1978). Redox potentiometry: determination of midpoint potentials of oxidation-reduction components of biological electron-transfer systems. *Methods Enzymol***54**, 411-35.
78. Wilson, G. S. (1978). Determination of oxidation-reduction potentials. *Methods Enzymol***54**, 396-410.

79. Pandelia, M. E., Ogata, H., Currell, L. J., Flores, M. & Lubitz, W. (2010). Inhibition of the [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F by carbon monoxide: an FTIR and EPR spectroscopic study. *Biochim Biophys Acta***1797**, 304-13.
80. Knüttel, K., Schneider, K., Erkens, A., Plass, W., Müller, A., Bill, E. & Trautwein, A. X. (1994). Redox properties of the metal centers in the membrane-bound hydrogenase from *Alcaligenes eutrophus* CH34. *Bull Pol Acad Sci Chem***42**, 495-511.
81. Asso, G., Guigliarelli, B., Yagi, T. & Bertrand, P. (1992). EPR and redox properties of *Desulfovibrio vulgaris* Miyazaki hydrogenase: comparison with the Ni-Fe enzyme from *Desulfovibrio gigas* *Biochim Biophys Acta***1122**, 50-56.
82. Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J., LeGall, J., DerVartanian, D. V., Peck, H. D., Jr. & Huynh, B. H. (1989). Redox intermediates of *Desulfovibrio gigas* [NiFe] hydrogenase generated under hydrogen. Mossbauer and EPR characterization of the metal centers. *J Biol Chem***264**, 16435-50.

Figure legends

Fig. 1. Structure of the FrhABG complex. The subunits FrhA, FrhB and FrhG are shown in blue, orange and green. The Ni, Fe and S of the [NiFe]-centers and the [4Fe-4S] cluster are shown as green, brown and yellow spheres and FAD as stick model (C, N and O in grey, blue, red). (a) The FrhABG protomer indicates the relative arrangement of the three subunits and the redox cofactors. (b) Surface representation of the dodecamer viewed along the threefold axis where three FrhA subunits of three different FrhABG dimers assemble. A narrow pore penetrable for water molecules is formed between the FrhA subunits at four vertices of the cube. (c) Three FrhB subunits coincide at the other four vertices of the FrhABG complex but do not contact each other. A pore with a diameter of 8 Å is formed across the protein shell.

Fig. 2. Structure of FrhA and its [NiFe]-center. (a) Fold. Terminal domains I and II, the four-helix bundle and the insertion domains are drawn in blue, lightgrey, lightblue and grey. The [NiFe]-center is located between the four domains. A metal ion, presumably a Mg^{2+} ion (wheat), sits near the protein surface. (b) The [NiFe]-center of the “as isolated” FrhABG and its $2F_o - F_c$ electron density map (contour level 2.0σ). The distance between Ni (in green) and Fe (in brown) is 2.75 Å. The Fe is most probably ligated with one CO and two CN^- ligands. (c) Surrounding of the [NiFe]-center. The superimposed FrhA and *D. gigas* [NiFe]-hydrogenase are drawn as stick model with the carbons in lightblue and grey.

Fig. 3. Structure of FrhG carrying three [4Fe-4S] clusters. (a) Fold. FrhG is composed of an N-terminal (lightgreen) and a C-terminal (green) domain. The N-terminal domain of FrhG is superimposed with the related small subunit of group 1 [NiFe]-hydrogenase of *D. gigas* (PDB-code; 1frv, in grey). The fold of the C-terminal domain of FrhG and the positions of the medial and distal [4Fe-4S] clusters differ between group 1 and 3 enzymes. (b) Proximal [4Fe-4S] cluster bound to the N-terminal domain of FrhG with $2F_o - F_c$ electron density (contour level 2.0σ). The proximal [4Fe-4S] cluster is coordinated with Cys57, Cys132, Cys171 and Asp60. (c) Surrounding of the proximal [4Fe-4S] cluster. FrhG (green) and *D. gigas* [Ni,Fe] hydrogenase (grey) are superimposed.

Fig. 4. Structure of FrhB. (a) Fold. The **ab** core region is drawn in orange, the inserted helix-loop-helix region in yellow, the inserted **b**-meander subunit in brown and the long **a**-helix in red. (b) The FAD binding mode. The U-shaped FAD is embedded into a pocket with a size of about $14 \text{ \AA} \times 13 \text{ \AA} \times 7 \text{ \AA}$. The *Si*-face of the isoalloxazine ring points towards the frontside. Residues that form polar interactions with FAD are included. (c) The [4Fe-4S] cluster of FrhB. The irons are coordinated with Cysb104, Cysb134, Cysb192 and Cysb195 and embedded into a positively charged surrounding. A loop segment of FrhG is drawn in green.

Fig. 5. The most attractive H₂ and proton pathways. FrhA and FrhG are colored in light blue and light green, respectively. Amino acids are shown in sticks. (a) A hydrophobic channel (shown as orange surface) extends from the buried [NiFe]-center to the protein surface over $20 \text{ \AA}$ and is suitable for H₂ transport. (b) Postulated proton transfer route from Glu^a14, via His^a53 and several firmly bound water molecules (electron density in green; contoured at 2σ).

Graphical Abstract

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5