Importance of the Protein Framework for Catalytic Activity of [FeFe]-Hydrogenases

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Background: Hydrogenases contain a unique oxygen-labile metal cofactor.

Results: Substitution of noncovalently interacting residues degrades the catalytic cofactor (K358N and M497L) or reduces activity but leaves the cofactor chemically intact (C299S and M353L).

Conclusion: Lys358 and Met353 are essential for H-cluster coordination. Cys299 and Met353 influence catalytic activity only.

Significance: Understanding specific cofactor-amino acid interactions provides an important basis for improving artificial hydrogen catalysts.

The active center (H-cluster) of [FeFe]-hydrogenases is embedded into a hydrophobic pocket within the protein. We analyzed several amino acids, located in the vicinity of this niche, by site-directed mutagenesis of the [FeFe]-hydrogenases from Clostridium pasteurianum (Cpl) and Chlamydomonas reinhardtii (CrHydA1). These amino acids are highly conserved and predicted to be involved in H-cluster coordination. Characterization of two hydrogenase variants confirmed this hypothesis. The exchange of residues CrHydA1Met415 and CrHydA1Lys228 resulted in inactive proteins, which, according to EPR and FTIR analyses, contain no intact H-cluster. However, [FeFe]-hydrogenases in which CplMet353 (CrHydA1Met223) and CplCys299 (CrHydA1Cys169) were exchanged to leucine and serine, respectively, showed a structurally intact H-cluster with catalytic activity either absent (CplC299S) or strongly diminished (CplM353L). In the case of CrHydA1C169S, the H-cluster was trapped in an inactive state exhibiting g values and vibrational frequencies that resembled the Htrans state of Desulfovibrio desulfuricans. This cysteine residue, interacting with the bridge head nitrogen of the di(methyl)amine ligand, seems therefore to represent an essential contribution of the immediate protein environment to the reaction mechanism. Exchanging methionine CplMet353 (CrHydA1M223) to leucine led to a strong decrease in turnover without affecting the KM value of the electron donor. We suggest that this methionine constitutes a “fine-tuning” element of hydrogenase activity.

Hydrogenases are complex catalysts for H2 oxidation and proton reduction (1), which include at their active sites unique “organometallic” cofactors containing either iron only or iron and nickel. Depending on the metal content of the cofactor, hydrogenases are assigned to three main classes, namely [FeFe]-, [NiFe]-, and [Fe]-hydrogenases (also known as Hmd) (2–4). Although these three classes of hydrogenase are phylogenetically distinct and display structural differences, they have in common the coordination of the iron ions of the active sites by unusual non-protein CO and CN ligands (1,5,6) that stabilize low spin and low oxidation states of the iron ions (7–9).

X-ray structural data for two bacterial [FeFe]-hydrogenases, which reveal the molecular arrangement of their active sites, have been available since 1998 (10,11). Results of different spectroscopic methods indicate that the active sites of all [FeFe]-hydrogenases feature the same molecular assembly (7,12–14). This prosthetic group, the so-called H-cluster, consists of a [4Fe-4S] subcluster and a [2Fe-2S] subcluster, which are connected by a cysteine-sulfur from the protein environment. Each iron ion of the [2Fe] subcluster is coordinated by one CN and one CO ligand. A third CO group is in a bridging position between both iron ions. The two sulfur atoms of the [2Fe] subcluster are connected by a dithiolate bridge ligand, the identity of which is now considered to be di(methyl)amine, i.e. with a central nitrogen atom (15–20). Depending on their position relative to the [4Fe] subcluster, the iron ions of the [2Fe] subcluster are referred to as being “proximal” or “distal.” The proximal iron is connected to the [4Fe] subcluster by a cysteine, whereas the distal iron harbors an open coordination site. The activity of the H-cluster is inhibited in the presence of free CO in the solution. In this case, an additional CO ligand occupies the open coordination site at the distal iron, forming an EPR-active CO-inhibited state (Hox-CO) (12,21). This additional CO ligand dissociates under illumination at cryogenic temperatures below 60 K. The resulting state of such photodissociation is the active oxidized state (Hox) (8,12,22). For a summary of [FeFe]-hydrogenase redox states, see supplemental Fig. S1.

Having this structural information available, chemical model molecules resembling the H-cluster framework have been synthesized with the aim of creating catalysts able to perform H2 generation without the need for noble metals like platinum. Although several chemical mimics catalyze electrochemical
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reduction of protons, none of them works as fast and at a similarly mild potential as their natural counterparts (23). In contrast they require an input of energy well beyond the thermodynamic minimum; thus they operate only at large overpotentials; furthermore, they are stable only in certain non-aqueous solvents such as CH₂CN, whereupon H₂ evolution takes place upon the addition of an acid (24).

These differences can be explained taking into account a catalytic mechanism of hydrogenases, in which a proton binds to the [2Fe] subcluster and has to be reduced to an intermediate hydride. In a 2Fe unit, this hydride could in principle be formed in a terminal position at one iron atom or in a position bridging both iron atoms, calculated to be thermodynamically more stable (16, 17, 25–29). Although the majority of artificial [FeFe]-hydrogenase model compounds feature hydrides in a bridging position (24), terminal protonation is proposed to be essential for fast catalysis (1, 30–32). For synthetic [FeFe] analogs it has been shown that to promote terminal protonation, the center should be in a thermodynamically unfavored “rotated” conformation (24, 33). In the enzyme this configuration is probably stabilized by a number of first and second sphere interactions (15, 34), indicating the importance of the surrounding protein environment for efficient catalytic activity.

The H-cluster is located in a conserved hydrophobic pocket within the protein structure. This pocket (so called H-domain) is the most conserved part of [FeFe]-hydrogenases, as observed in multiple amino acid sequence alignments (supplemental Fig. S2). The [4Fe] subcluster is covalently bound to the peptide chain by four cysteines, and one of these connects it to the [2Fe] subcluster. Furthermore, a number of conserved amino acids are located close to the different ligands of the H-cluster and may stabilize its unique geometry. Herein we have analyzed the importance of four of these conserved amino acids by site-directed mutagenesis of two different [FeFe]-hydrogenases and characterized the resulting eight variants by subsequent biochemical and biophysical techniques. We show that C2995S and C169S exchanges in the [FeFe]-hydrogenases from Clostridium pasteurianum (termed Cpl in the following text) and Chlamydomonas reinhardtii (CrHydA1), respectively, modulate the electronic properties of the H-cluster while leaving it structurally intact. A methionine (CpIMet353/CrHydA1Met353) is crucial for the high turnover rate.

EXPERIMENTAL PROCEDURES

Multiple Sequence Alignment—We used BLAST to identify homologous proteins to Cpl in different species and created a multiple sequence alignment (MSA) using COBALT (35). Sequences missing two or more H-cluster coordinating cysteines were excluded from the MSA. This MSA (supplemental Fig. S1) together with the structure of Cpl (11) was used to calculate the evolutionary conservation of amino acid positions in Cpl using CONSURF (Fig. 1) (36–38).

Plasmids and Genetic Construction—The expression vector for overexpression of CrHydA1 in Clostridium acetobutylicum has been described previously (39). To construct and clone the expression vector pThydA<sub>Cp</sub>-C<sub>Tag</sub><sup>exp</sup>, a 5-kbp fragment was excised from the plasmid pThydA<sub>Cr</sub>-opt-C<sub>Tag</sub><sup>exp</sup> using the restrictionendonucleases BamHI and EcoRV. hydA<sub>Cp</sub> was amplified using the primers Cpl_BamHI_UP 5′-CCGG-ATCCAAAACAATTATATAATGTGACAG3′ and Cpl_EcoRV_DOWN 5′-CCGGATATCTTTTATACATGAG-3′, yielding a 1.7-kbp fragment. Digestion with BamHI and EcoRV allowed direct ligation to construct pThydA<sub>Cp</sub>-C<sub>Tag</sub><sup>exp</sup>. Site-directed mutagenesis was performed according to the protocol described by Zheng et al. (40). Competent Escherichia coli DH5α cells were used for all genetic construction experiments, and all plasmids were sequenced before being transferred into C. acetobutylicum MGCCac15 (41). C. acetobutylicum recombinant strains were stored in spore form at −20 °C and were stable for several months.

Purification of Strep Tag II-tagged [FeFe]-Hydrogenases—C. acetobutylicum MGCCac15 recombinant strains were grown anaerobically in complete growth medium containing up to 60 g/liter glucose in a 2.5-liter MiniFors bioreactor (Infors, Zug, Switzerland). Protein purification was performed under strictly anoxic conditions (39, 42) by a one-step purification protocol. Affinity chromatography on a 2-mL Strep-Tactin Superflow® (IBA Göttingen, Germany) was carried out using 100 mM Tris/HCl, pH 8.0, as buffer, 2 mM dithionite as reducing agent, and 2.5 mM desthiobiotin for elution of the protein.

Biochemical Characterization—H₂-evolving activity was determined by in vitro assays with 10 mM methyl viologen as the electron donor as described previously (44). The gas mixture was injected into a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) equipped with a PLOT fused silica coating Molecular sieve column (5 Å, 10 m × 0.32 mm) from Varian (Palo Alto, CA). The specific activity of the hydrogenase was calculated from the detected amount of produced H₂. The protein concentration was determined by the Bradford assay (45) using a Bradford reagent obtained from Bio-Rad. Kinetic parameters V<sub>max</sub> and K<sub>m</sub> were calculated via Lineweaver-Burk plots of the H₂ production rate with methyl viologen concentrations varying from 0.5 to 20 mM.

H₂ oxidation activity was determined by a method based on that described by Adams and Mortenson (46). The assay mixture contained 0.05 mM methylene blue in 100 mM Tris/HCl, pH 8.0, under 2% H₂ at 30 °C in a total volume of 200 μl. The reaction was initiated by adding 50 ng to 6.4 μg of hydrogenase, and oxidation of methylene blue was followed with a Beckman Coulter PARADIGM™ absorbance detection cartridge at 604 nm.

Biophysical Characterization—Q-band EPR spectra of all samples were measured by using the 2-pulse electron spin echo-detected EPR technique (47) using a Bruker ELEXSYS E580 setup as described previously (48). Fourier transform IR (FTIR) measurements were performed on a Bruker IFS 66 v/s FTIR spectrometer equipped with a Bruker MCT (mercury-cadmium-telluride) detector (48). If not specified, the temperature was set at 200 K. The interferograms were accumulated in the double-sided, forward-backward
mode with 2000 or more scans. All FTIR spectra were obtained with 3 cm⁻¹ resolution.

Treatment with CO was performed in a gas-tight glass vial flushing pure CO gas through as described previously (48). Thionine treatment was carried out by small additions of a highly concentrated anaerobic solution of thionine to a final concentration of about 2 mM. The oxidation was controlled by monitoring the disappearance of the blue color (reduction of thionine). After oxidation, the solution was transferred to an EPR tube and frozen within 2 min in liquid nitrogen (77 K).

Electrochemical Characterization—Protein film electrochemistry experiments were carried out in an anaerobic glove box as described previously (49). Experiments were performed in phosphate buffer titrated to the desired pH at the experimental temperature. A pyrolytic graphite edge rotating disc electrode was used with an electrode rotator (EcoChemie) fitted into a gas-tight glass electrochemical cell as described previously (50). Potentials are quoted relative to the standard hydrogen electrode (SHE) using the correction \( E_{\text{SHE}} = E_{\text{SCE}} + 0.242 \text{ mV at 298 K} \), where SCE is saturated calomel electrode. Mass flow controllers were used to prepare precise gas mixtures and to impose constant gas flow rates into the electrochemical cell during experiments. Efficient mixing and gas-solution equilibration were achieved through rapid electrode rotation (3000 rpm), which also ensured an efficient supply of substrate and removal of product. Enzyme films were prepared using the method described by Wait et al. (49) using 1.5 µl of a 2 g/liter enzyme solution. Cyclic voltammograms were recorded under 100% \( \text{H}_2 \) (1000 standard cm³/min (sccm)) at a scan rate of 20 mV/s. Electrochemistry experiments to determine the \( K_m \) for \( \text{H}_2 \) oxidation were conducted at \(-0.05 \text{ V (SHE)} \) at 10 °C by varying the ratio of \( \text{H}_2 \) to \( \text{N}_2 \) in the cell head space using mass flow controllers. Values for \( K_m \) were calculated from the \( x \)-intercept of a plot of 1/current against 1/hydrogen concentration (52).

RESULTS AND DISCUSSION

Amino Acid Residues Forming the H-cluster Pocket Are Highly Conserved—An MSA of 408 sequences with high similarity to Cpl was generated to analyze the conservation levels of the first sphere interaction residues of the 2Fe subunit of [FeFe]-hydrogenases. The amino acid positions were scored from 1 (low conservation) to 10 (high conservation). In total, 98 of 574 amino acids showed the highest level of conservation (level 10) (Fig. 1). Almost all highly conserved residues are localized in the part of the H-domain surrounding the H-cluster, and most have a predictable structural relevance (for example 17 cysteines that bind the [FeS] clusters to the protein) (Fig. 1). Eight amino acids are situated in close proximity to the [2Fe] subcluster and might thus be important for stabilizing the orientation of the active site. In the case of the hydrogenase Cpl from \textit{C. pasteurianum}, these residues and suggested interactions are: Pro321 and Pro324, which have a N-N electrostatic interaction with the terminal and proximal CN group, respectively; Ser325, which forms a hydrogen bond to the proximal CN group; Gln325 and Lys358, which form a hydrogen bond to the distal CN group; Met397 and Cys299, which hydrogen bond to the dithiolate bridging ligand; and finally Met353, which forms an S-O electrostatic interaction to the bridging CO (34). Apart from Met353 and Gln325, which were frequently exchanged for other amino acids, these residues showed only few substitutions among other [FeFe]-hydrogenases (Table 1). Four of the...
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TABLE 1
Conservation of the H-cluster vicinity
Amino acid residues of Cpl with noncovalent interactions to the [2Fe] subcluster are presented. Distances estimated from Cpl and DdH crystal structures are between interacting atoms (sulfur, nitrogen, and oxygen).

| Cpl residue | Bonding atom | Distances \( d_{\text{Cpl}} \) \( d_{\text{DdH}} \) | Substitutions in MSAa | Variants in SDM\(^{b}\) analysis |
|-------------|--------------|----------------------|-------------------|----------------------|
| Pro\(^{231}\) | Proximal CN | 3.6 3.5 | Ser (1) | C299S C169S |
| Ser\(^{232}\) | Proximal CN | 3.1 2.9 | Ala (327), Phe (1) | M353L M223L |
| Cys\(^{299}\) | Dithiolate N | 3.5 3.2 | Ser (1), Trp (1), Phe (1) | K358N K228N |
| Pro\(^{234}\) | Distal CN | 3.5 3.6 | Cys (7) | M497L M415L |
| Glu\(^{325}\) | Distal CN | 2.9 3.0 | His (47), Met (38), Ile (56), Leu (4), Val (3) | |
| Met\(^{353}\) | Bridging CO | 3.1 3.4 | Thr (30), Val (5), Asn (5), Leu (2), Gln (1), Ala (1), Ile (1), Gly (1) | |
| Lys\(^{358}\) | Dithiolate N | 2.9 2.9 | Asn (1), Ala (1) | |
| Met\(^{417}\) | Dithiolate N | 3.6 3.9 | Val (2), Leu (1) | |

a Numbers in parentheses mark the quantity of respective substitutions in the MSA (supplemental Fig. S2).
b Site-directed mutagenesis.
\( ^{c} \) Ref. 11.
\( ^{d} \) Ref. 10.

above mentioned eight amino acids have been proposed to interact with the [2Fe] subcluster via the amino groups of the polypeptide backbone (34) (Fig. 1 and Table 1). In these cases a single amino acid exchange would not necessarily result in an interruption of the respective interaction. The site-directed mutagenesis analyses of this study were therefore concentrated on residues Cys\(^{299}\), Met\(^{353}\), Lys\(^{358}\), and Met\(^{417}\) in Cpl and the corresponding positions in CrHydA1. In these cases, the interaction to the H-cluster ligands was assumed to originate from the nitrogen or sulfur atoms of the side chains and therefore to be specific for the single amino acid residues.

To minimize the structural effects in the resulting variants, the amino acids were mutated as conservatively as possible. Substitutions were chosen based on high scores as indicated by PAM1/BLOSSOM62 evolutionary distance matrices (53, 54). To determine whether any exchange effects could be referred to [FeFe]-hydrogenases in general rather than being specific for individual enzymes, we introduced mutations into both a eukaryotic [FeFe]-hydrogenase (Cpl) and a prokaryotic [FeFe]-hydrogenase (Cpl). The protein variants generated were C169S, M353L, K228N, and M497L (Table 1). In FTIR spectroscopy analyses, no signals were detected in the region of the CO/CN-stretching vibrations (1780–2110 cm\(^{-1}\)), which could be assigned to the diatomic ligands (not shown). By applying EPR spectroscopy to a CO-flushed sample of this variant, no H-cluster signal was visible. On the other hand, when the variant K228N was reduced by sodium dithionite (NaDT), EPR spectroscopy showed a clear rhombic spectrum with characteristic \( g \) values of 2.043, 1.915, and 1.900 (Fig. 2D), which are typical for [4Fe-4S] clusters. Moreover, these signals strongly resemble the EPR spectrum of the inactive form of CrHydA1 expressed without maturation factors in \( E. coli \) (48, 56) (Fig. 2H). This form lacks the 2Fe moiety, but the [4Fe] subcluster is intact. The absence of the [2Fe] subcluster in the CrHydA1K228N variant supports the theory that Lys\(^{228}\) (CplLys\(^{358}\)) is important for the orientation of the metal cluster during the maturation process (57). However, we observed a significant difference in the low field value between the EPR spectra of unmaturated and CrHydA1K228N species (Fig. 2H). The reason for this difference was unclear, but a possible explanation could be different conformations of the peptide chain that were proposed for the mature and immature forms (57). The hydrogen bond of CrHydA1Lys\(^{228}\) to the distal CN group represents a very strong noncovalent interaction of the H-cluster with its surrounding peptide (58). Based on the results of a quantum mechanics/molecular mechanics (QM/MM) analysis, this hydrogen bond is suggested to prohibit isomerization of the CO/CN ligands at the distal iron ion (58) and therefore to stabilize the H-cluster in a form in which the vacant coordination site is in the \( trans \) position to the bridging CO (59). This coordination geometry was in fact calculated to be more stable (by 73 kJ mol\(^{-1}\)) than the alternative geometry (58). This stability difference might also be a reason for the loss of the [2Fe] subcluster in the variant.

Interrupting Amino Acid Interactions with the Dithiolate Ligand Has a Strong Impact on H-cluster Activity and Structure—The dithiolate bridging ligand is a unique structural feature of [FeFe]-hydrogenases. Analyses of the available x-ray crystallographic data indicate its possible interaction with several neighboring amino acid residues (19, 34). In this study we individually exchanged two of these amino acids, CplMet\(^{497}\) and

Furthermore, no intact H-cluster could be detected in CrHydA1K228N. In FTIR spectroscopy analyses, no signals were detected in the region of the CO/CN-stretching vibrations (1780–2110 cm\(^{-1}\)), which could be assigned to the diatomic ligands (not shown). By applying EPR spectroscopy to a CO-flushed sample of this variant, no H-cluster signal was visible. On the other hand, when the variant K228N was reduced by sodium dithionite (NaDT), EPR spectroscopy showed a clear rhombic spectrum with characteristic \( g \) values of 2.043, 1.915, and 1.900 (Fig. 2D), which are typical for [4Fe-4S] clusters. Moreover, these signals strongly resemble the EPR spectrum of the inactive form of CrHydA1 expressed without maturation factors in \( E. coli \) (48, 56) (Fig. 2H). This form lacks the 2Fe moiety, but the [4Fe] subcluster is intact. The absence of the [2Fe] subcluster in the CrHydA1K228N variant supports the theory that Lys\(^{228}\) (CplLys\(^{358}\)) is important for the orientation of the metal cluster during the maturation process (57). However, we observed a significant difference in the low field value between the EPR spectra of unmaturated and CrHydA1K228N species (Fig. 2H). The reason for this difference was unclear, but a possible explanation could be different conformations of the peptide chain that were proposed for the mature and immature forms (57). The hydrogen bond of CrHydA1Lys\(^{228}\) to the distal CN group represents a very strong noncovalent interaction of the H-cluster with its surrounding peptide (58). Based on the results of a quantum mechanics/molecular mechanics (QM/MM) analysis, this hydrogen bond is suggested to prohibit isomerization of the CO/CN ligands at the distal iron ion (58) and therefore to stabilize the H-cluster in a form in which the vacant coordination site is in the \( trans \) position to the bridging CO (59). This coordination geometry was in fact calculated to be more stable (by 73 kJ mol\(^{-1}\)) than the alternative geometry (58). This stability difference might also be a reason for the loss of the [2Fe] subcluster in the variant.

The Hydrogen Bond Formed by Lys\(^{358}\)/Lys\(^{228}\) Is Essential for the Integrity of the [2Fe] Subcluster—The NH–N hydrogen bond between CplLys\(^{358}\) and the distal CN group (Fig. 1) has a N–N distance of 2.9 Å in Cpl and DdH (Table 1) and therefore lies in the typical range of peptide hydrogen bonds (55). It is worth noting that this strong NH–N bond is also evident from the \( ^{14} \)N signal of the lysine side chain in an EPR study of DdH (19). This amino acid residue is highly conserved, and only two [FeFe]-hydrogenases are found to have different amino acids (Asn or Ala) at this position (Table 1). Both of these are protozoan proteins that have not yet been shown to be functional hydrogenases. In the current study, the importance of the hydrogen bond formed by this lysine residue (Lys\(^{358}\) in Cpl and Lys\(^{228}\) in CrHydA1) was analyzed by exchanging it for asparagine and thereby interrupting the hydrogen bond. The resulting protein variants, CplK358N and CrHydA1K228N, lacked any measurable hydrogenase activity, as they were unable either to reduce protons or to oxidize \( H_2 \) (Table 2).
CpICys$^{299}$ (Fig. 1). A clear specification of the interactions is difficult because of the possible different protonation states of the dithiolate amine nitrogen ($^{19}$) and CpICys$^{299}$. Furthermore, the protonated NH$_2$/H$^{+}$ form (Fig. 1) was calculated to be more stable in a different steric conformation as observed in x-ray crystal structures with the amine directed toward CpICys$^{503}$.

### TABLE 2

Activity in proton reduction and hydrogen oxidation of CrHydA1 and CpI variants

Each value represents the mean of at least three different measurements. ND, not determined.

| Variant   | Proton reduction | Hydrogen oxidation |
|-----------|------------------|--------------------|
|           | $K_m$ μmol methyl viologen | $V_{max}$ μmol H$_2$ min$^{-1}$ mg$^{-1}$ | % | $K_m$ μmol methylene blue min$^{-1}$ mg$^{-1}$ | % |
| CrHydA1   |                  |                    |    |                  |    |
| WT        | 766               | 730$^a$            | 100| 0.377            | 6.1 ± 1.2 | 100 |
| M223L     | 663 ± 180         | 112 ± 7            | 15 | 0.241            | 4.2 ± 0.9 | 68  |
| K228N     | Inactive         | Inactive           | 0  | ND               | Inactive | 0   |
| C169S     | Inactive         | Inactive           | 0  | ND               | Inactive | 0   |
| M415L     | 690 ± 76         | 33 ± 4             | 5  | ND               | 0.9 ± 0.2 | 14  |
| CpI       |                  |                    |    |                  |    |
| WT        | 1779 ± 353       | 1598 ± 103         | 100| ND               | 3.1 ± 0.4 | 100 |
| M353L     | 1800 ± 292       | 248 ± 43           | 15 | ND               | 2.3 ± 0.3 | 74  |
| K358N     | Inactive         | Inactive           | 0  | ND               | Inactive | 0   |
| C299S     | Inactive         | Inactive           | 0  | ND               | Inactive | 0   |
| M497L     | 1026 ± 47        | 67 ± 21            | 4  | ND               | 0.8 ± 0.4 | 25  |

$^a$ Ref. 75.

### FIGURE 2

**Q-band pulse EPR spectra of variants of CrHydA1.** Respective g-tensor components are marked by arrows. A, CrHydA1 wild type H$_2$–CO state. B, CrHydA1M223L flushed with CO. C, CrHydA1M415L flushed with CO (black); CrHydA1M415L oxidized with thionine (gray). Dashed line, difference spectrum of the black and gray spectra. D, CrHydA1K228N reduced with 10 mM sodium dithionite. E, CrHydA1M223L reduced with 2 mM sodium dithionite. F, CrHydA1M415L reduced with 2 mM sodium dithionite. G, CrHydA1C169S reduced with 10 mM sodium dithionite. H, overview of measured g values. $^*$, difference spectrum of panel C. Footnote 1, see Ref. 13; Footnote 2, see Ref. 12; Footnote 3, see Ref. 48.
which in principle could allow hydrogen bonding to CpIMet\textsuperscript{497}. Hence, CpICys\textsuperscript{299} could form a SH—N or S—HN hydrogen bond and CpIMet\textsuperscript{497} a N—HS hydrogen bond. According to CpI and DdH crystal structures, the S—N distances are 3.6 Å/3.9 Å in the case of CpIMet\textsuperscript{497} and 3.5/3.2 Å in the case of CpICys\textsuperscript{299} (Table 1). These distances are long compared with the typical protein hydrogen bonds and are therefore assumed to constitute weaker interactions than those of CpILys\textsuperscript{358} and the distal CN ligand. Nevertheless, both amino acids were shown to be highly conserved in the MSA of CpI homologues. For either position, only three cases of substitution were found via the alignment (Table 1). All of these occur in uncharacterized putative hydrogenases, mostly of protozoan species.

The CpIM497L variant showed a strongly diminished proton reduction activity, which was only 4% of the activity of the wild type enzyme. The same loss of activity (5% residual) was exhibited by the corresponding variant (M415L) of CrHydA\textsubscript{1} (Table 2). H\textsubscript{2} oxidation activity was also strongly decreased in both variants (Table 2).

Interestingly, a NaDT reduced sample of CrHydA\textsubscript{1}M415L showed a rhombic EPR spectrum with g values of 2.098, 2.031, 2.003, 1.958, and 1.890 (Fig. 2C), which are quite different from CrHydA\textsubscript{1} wild type g values. These results indicate that the exchange from methionine to leucine strongly affects the electronic structure and/or architecture of the H-cluster. The detected low field and middle field g values of 2.098 and 2.031 are similar to (although not quite the same as) the H\textsubscript{ox} state, but the high field values were different from any known g values obtained using wild type proteins. A comparison of this spectrum with that of a thionine-oxidized sample (Fig. 2C) further identified an axial spectrum highly similar to H\textsubscript{ox}-CO of the wild type protein (Fig. 2, C and H). We conclude therefore that the overall spectrum consists of three different components: CrHydA\textsubscript{1}M415L H\textsubscript{ox}, 2.098–2.031; H\textsubscript{ox}-CO, 2.049–2.007; and an unknown species of 1.958–1.890. Based on the character and the characteristic g values of the high field shoulder, it is likely that the unknown signals correspond to a [4Fe-4S]-type cluster. However, as the signal is rather small in comparison with the rest of the EPR spectrum, we prefer to avoid further speculations about the possible nature of this signal and consider it as a minor impurity. FTIR analyses showed two different sets of bands (Fig. 3A).

The main contributing signals did not shift during illumination. These bands occurred at wave numbers of 2094, 2030, 1986, 1933, and 1804 cm\textsuperscript{-1}, rather unusual positions of which the
nature is unclear. Accounting for the number of visible bands and their position, we can speculate that these light-independent IR signals correspond to a sort of $H_{\infty}$-CO state with disrupted geometry, as evident from the unusually low position of one of the CN bands (2030 cm$^{-1}$). Probably, this distortion locks the $H_{\infty}$-CO state and makes it impossible to dissociate the CO ligands. The other set of bands at 2088, 2081, 2014, 1969, 1961, and 1806 cm$^{-1}$ was replaced by bands at 1964, 1940, and 1796 cm$^{-1}$ upon illumination. These signals are highly similar to the bands of CrHydA1 wild type enzyme measured under similar conditions (Fig. 3E) and thus were assigned to the signals of the $H_{\infty}$-CO and the $H_{\infty}$ states, respectively. The FTIR signals are rather weak in contrast to the strong signals of the [4Fe] subcluster observed in EPR under reducing conditions (note the signal-to-noise ratio as compared with other spectra on Figs. 2 and 3). We repeated the experiments, including activity tests, several times with completely consistent results. Hence, it is evident that CrHydA1M415L is predominantly inactive, containing only a [4Fe] subcluster, whereas there is a minor fraction that contains a complete 6Fe assembly.

A possible explanation could be the existence of several protein conformations induced by mutation of this methionine to leucine. The mutation from methionine to leucine is a structurally highly conservative mutation, which in multiple cases has been shown to have no effect on the overall protein structure (60, 61). However, the side chain of methionine is considered to have a high conformational flexibility compared with leucine (62). The C–S bond of methionine is rather long and has a low energy barrier to rotation about this bond (63). Consequently, various configurations for the methionine side chain are possible at room temperature and indeed have been observed in protein crystal structures (64). Because of these characteristics, methionines were expected to be important for the binding of differently dimensioned partners to one specific binding site. Methionine-rich surfaces should be malleable and adapt themselves to the binding partner (61). In hydrogenases, CrHydA1Met$^{415}$ might in a similar way be a “buffer” between the inorganic cofactor and the peptide framework, cushioning conflicts that result from relative movements of both species and sustaining the hydrophobic cavity. A decrease of this buffering function might explain the degradation of the [2Fe] subcluster in the main part of CrHydA1M415L.

In contrast to CrHydA1M415L and CplM497L, variants CrHydA1C169S and CplC299S had no $H_2$ evolution or $H_2$ oxidation activity at all (Table 2). Furthermore, no EPR signals could be observed when the sample of CrHydA1C169S was flushed with CO (not shown). On the other hand, a NaDT reduced sample of this variant featured a rhombic spectrum with g values of 2.067, 1.941, and 1.880 (Fig. 2G). These are quite different from all of the spectra observed for CrHydA1 wild type proteins but resemble the g values determined for quite different from all of the spectra observed for CrHydA1 (active) and the [2Fe] subcluster in a “superoxidized” form, Fe(II)-Fe(II) (67). To our knowledge, this state has never been observed before in CrHydA1 or Cpl.

The FTIR spectrum of this sample showed bands at 2106, 2090, 2079, 2074, 1983, 1977, 1968, 1960, 1948, 1867, and 1857 cm$^{-1}$ (Fig. 3D), which indicated a mixture of at least two different states. Because only one state was detected via EPR spectroscopy, it is most likely that one of the states is EPR-silent. This is supported by the fact that no EPR signal could be detected in the sample without NaDT (thus oxidized). Reduction/oxydation of the [2Fe] subcluster would considerably perturb the position of the CO/CN bands in the IR spectrum, which was not the case. Hence, the redox reaction in this mutant indeed happens in the [4Fe] subcluster, without affecting the [2Fe] subcluster. Four bands of the CrHydA1C169S spectrum (2106, 2074, 1883, and 1977 cm$^{-1}$) showed a high similarity to bands observed in the $H_{\text{trans}}$ state of DdH (7), thus confirming our assignment of the EPR signals. Two other bands were located in a part of the spectrum representing the bridging ligand within the [2Fe] subcluster, but they appeared at significantly higher wave numbers (Fig. 3E) than those normally observed in CrHydA1 wild type or DdH $H_{\text{trans}}$. This might indicate a semibridging position of the corresponding CO ligand or an unusual charge distribution in the [2Fe] subcluster.

It is believed that in the inactive states of DdH such as $H_{\text{trans}}$, an oxygen-derived species occupies the open coordination site at the distal iron (26, 68). This group might modify the redox properties of the H-cluster, allowing a water molecule or a hydroxide to bind tightly to the open coordination site and prevent activation. Additionally this configuration could be stabilized by direct interaction of the prospective water or hydroxide ligand to the introduced serine. Indeed hydrogen bonds to surrounding molecules have a strong influence on the competitive affinity of $H_2$O and $H_2$ for metal sites (69).

It is worth noting that another proposed function of the conserved cysteine residue, CplCys$^{299}$ (CrHydA1Cys$^{169}$) (Fig. 1), is that it might be part of a proton transfer pathway from the protein surface to the active site (1, 11). Although serine is well known to mediate proton transfer reactions in proteins, the mutation could isolate the H-cluster from the proton transfer pathway and thus inactivate the enzyme. Recently, however, two variants of HydA from C. acetobutylicum were reported, in which Cys$^{298}$, corresponding to CrHydA1Cys$^{169}$ and CplCys$^{299}$, was exchanged for alanine or leucine (70). Both variants exhibited $H_2$ oxidation activities between 15 and 20% of the wild type protein activity (70), whereas the CrHydA1C169S and CplC299S variants analyzed in this study had no activity at all. Based on these observations, we suggest that the introduction of an OH group results in a functional blocking of the catalytic events, whereas the introduction of aliphatic substituents just hinders the proton transfer.

It remains puzzling as to why DdH species can be obtained upon aerobic purification in an inactive state, whereas Cpl and CrHydA1 wild type species cannot be obtained. Possibly there is a fine detail in the surrounding of the H-cluster that allows or forbids this behavior, and the CrHydA1C169S mutation has Functions of Conserved Amino Acids in [FeFe]-Hydrogenases
triggered it. Ongoing characterization of the C169S will hopefully help to understand the basic relation. Whatever the reason, an important conclusion is that the C169S mutation, which is essentially just a substitution of SH to OH, results in a drastic modification of the properties of the H-cluster without destroying its integrity.

S–O Bonds between Methionine Met353/Met223 and the Bridging CO Influence the Direction of the Catalytic Reaction of [FeFe]-Hydrogenases—Only one of the five diatomic ligands of the [2Fe] subcluster is coordinated by both iron ions and is thus termed the bridging CO ligand. One conserved amino acid (Met353 in CpI) probably stabilizes this bridging CO by an S-O electrostatic interaction (Fig. 1) (34). Electrostatic dipole interactions are relatively weak and have a rather short distance range (71). The distances estimated between the relevant oxygen and nitrogen atoms in CpI and DdH crystal structures change significantly (3.1 and 3.4 Å, respectively) (Table 1). Thus, it is rather interesting to compare the results of corresponding mutations in these species. In this work, CpIMet353 and CrHydA1Met223 were exchanged for a structurally conservative leucine residue. Both CpIM353L and the corresponding CrHydA1M223L variant exhibited a strong decrease in H2 evolution activity, which was only 15% of the respective wild type activity (Table 2). In contrast, H2 oxidation activity as determined using oxidized methylene blue as the electron acceptor, was decreased less in both cases and reached activities of 68% (M223L) and 74% (M353L) compared with the corresponding wild type hydrogenases (Table 2).

The characteristics observed with artificial electron donors and electron acceptors of the enzyme variants might have resulted from a shift of the redox potentials or a higher affinity for H2. To check the relative activities for H2 evolution and H2 oxidation, the electrocatalytic activity of CrHydA1M223L was recorded with the enzyme adsorbed on a pyrolytic graphite electrode. Because of its lower catalytic activity, this protein variant had to be applied to the electrode in higher concentrations (2 g/liter) than the wild type hydrogenase (0.1 g/liter). The catalytic activity of CrHydA1M223L (Fig. 4, C–E) shows the typical bidirectional behavior of the wild type enzyme (Fig. 4B), as seen from the normalized cyclic voltammograms shown in Fig. 4A. In this case the voltammograms of CrHydA1M223L and the wild type are nearly identical, which suggests that they have a similar catalytic bias to operate in either direction (under 1 bar H2). Therefore the catalytic activity of the variant is decreased by the same amount in both, H2 oxidation and proton reduction activity, and the observed differences are most probably due to differences in the assay conditions such as different pH values. We further used the immobilized enzyme to determine the \( K_m \) value for H2 as described previously (52). The calculated \( K_m \) of the mutant was slightly lower but comparable.
with the wild type (Table 2), which might to some extent also explain the differences in results obtained with artificial electron donors and acceptors.

In EPR analyses, the CO-treated sample of CrHydA1M223L exhibited an axial spectrum with g values of 2.048 and 2.007 (Fig. 2B). The spectrum was very similar to the axial spectrum of a CO-inhibited wild type CrHydA1 sample with g values of 2.053 and 2.007 (Fig. 2, A and H). After illumination of the CO-inhibited M223L variant, a rhombic spectrum with g values of 2.102, 2.041, and 1.998 was observed, which was again highly similar to the CrHydA1 wild type spectrum recorded under similar conditions (Fig. 2H). These findings indicated that the structure of the H-cluster was intact, whereas the slightly altered g values hint at some slight differences in the electronic structure.

As analyzed by FTIR spectroscopy, the CO-purged sample of M223L exhibited bands at 2093, 2085, 2014, 1968, 1950, and 1803 cm⁻¹, which shifted during illumination to give another set of bands at 2073, 1986, 1933, 1914, and 1806 cm⁻¹ (Fig. 3B). These signals resembled the bands of CrHydA1 wild type enzyme under similar experimental conditions (Fig. 3E) (30). However, a shift of the bands representing the terminal and bridging CO ligands to lower wave numbers indicates a redistribution (increase) of charge around those CO ligands. In a sample of variant CrHydA1M223L treated with small amounts of NaDT (2 mM), another set of IR bands was detected at 2069, 2037, 2030, 1968, 1930, 1908, and 1881 cm⁻¹ (Fig. 3C). Even though this band pattern might have resulted from a mixture of different states, some of these bands can, with high likelihood, be assigned to the so-called H_red state of CrHydA1 (30). The EPR spectrum of this NaDT-treated sample showed a rhombic spectrum with g values 2.060, 1.920 and 1.850 (Fig. 2E). It had a [4Fe-4S]-like character and probably originated from the H-cluster in the H_red state. However, as no studies have been performed yet, further experiments are needed to clarify this point. Nevertheless, it is surprising that in CrHydA1M223L this state was detectable in an as isolated sample (2 mM NaDT), whereas in the wild type enzyme it has been thus far detected only by electrochemical titrations (30). It might be related to the electrostatic interaction of CplMet³⁵³ with the bridging CO ligand. This ligand was observed to shift between a bridging position and a terminal position either in the redox transition from H₄₋ to H₄⁺ (7, 8) or, in some proteins like CrHydA1, not before a further redox transition at lower potentials (H_red to H_red⁺) (30).

Consistent with measurements of CO-treated samples, a shift to lower wavelengths of some bands was observed in the NaDT-treated sample of CrHydA1M223L compared with the IR spectrum of the wild type CrHydA1. This shift indicates stronger bonding of the CO ligands, which might impede the transition between different redox states and possibly “slow down” the catalytic reaction rates. The detection of H_red under standard conditions in M223L indicates that the transition from H_red to H_red⁺ might be affected. This would explain the observed reduced activity.

It should be noted that the MSA conducted in this study showed that the position of this methionine residue (CrHydA1Met²²³) has a much higher variability than those of the other three conserved residues we chose to exchange in this study. This indicates that this residue is not essential for hydrogenase function. Instead, CrHydA1Met²²³ might be important for a high turnover rate of the enzyme. A total of 46 substitutions were identified in the MSA of 409 CrHydA1 homologues (Table 1). Most often (30×) methionine was exchanged for threonine, whereas valine (5×), asparagine (5×), and leucine (2×) were present at a lower frequency. The presence of threonine provides a side chain capable of forming an electrostatic O-O interaction with the bridging CO ligand. Most of the CrHydA1 homologues having, for example, threonine instead of methionine at the discussed position are found in bacteria or lower eukaryotes, which colonize higher organisms and are often found in the intestinal tract of human, cow, or sheep (supplemental Table 2). In this environment H₂ evolving organisms frequently live in symbiosis with methanogenic bacteria and transmit H₂ via so-called interspecies hydrogen transfer (73). Maintaining a low H₂ concentration in these environments is important for the thermodynamic equilibrium of the interacting metabolisms (74). A diminished catalytic activity of the [FeFe]-hydrogenases present in the H₂ evolving partners might be beneficial for controlling H₂ output. However, this hypothesis is at present based on theoretical considerations, as these hydrogenases have not yet been isolated and characterized.

Several structure-function relationships of [FeFe]-hydrogenases have already been investigated by applying site-directed mutagenesis. For example, the molecular interaction between CrHydA1 and its natural electron donor, ferredoxin PetF (photosynthetic electron transport ferredoxin), has been characterized by comparing the effects of different amino acid exchanges (75). Other analyses have been applied to generate variants with improved O₂ resistance in different hydrogenases (76–78). In this work we analyzed residues that were in close proximity to the H-cluster and likely to interact with its ligands. In summary, we have demonstrated the importance of highly conserved amino acids for the structural integrity of the H-cluster. Notably, two of these amino acids were further shown to be significant for H₂ evolution (catalytic) activity.

Nature has created hydrogenase enzymes as H₂-forming catalysts with a high turnover rate. However, they do not meet the demands of economically useable catalytic agents because of their limited stability and the cost of their production and purification. Artificial H-cluster analogues are promising alternatives for use in a future sustainable hydrogen economy. Most models of the hydrogenase active site reported thus far represent mimics of the inorganic cofactor only and show low H₂ evolution activity. To circumvent these restrictions, some general approaches to imitate the protein surrounding have been published (51, 72, 79 – 82). From the results shown in this study, we conclude that it may be possible and necessary to tailor these protein substitutes by specifically mimicking amino acids that support the catalytic activity of the H-cluster. Residues such as CplMet³⁵³, which influences hydrogenase turnover, or CplCys²⁹⁹, which is important to maintain the H-cluster in an active state, are promising targets for the generation of optimized hydrogenase model compounds.
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