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[Fe-Fe]-hydrogenase Reactivated by Residue Mutations as Bridging Carbonyl Rearranges: A QM/MM Study

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Abstract

In the current work, we found aqueous enzyme phase reaction pathways for the reactivation of the exogenously inhibited [Fe-Fe]-hydrogenases by O₂, or OH⁻, which metabolizes to H₂O^{1,2}. We used the hybrid quantum mechanics/molecular mechanics (QM/MM) method to study the reactivation pathways of the exogenously inhibited enzyme matrix. The ONIOM calculations performed on the enzyme agree with experimental results³, i.e., wild-type [Fe-Fe]-hydrogenase H-cluster is inhibited by oxygen metabolites. An enzyme spherical region with a radius of 8 Å (from the distal iron, Fe_d) has been screened for residues that prevent H₂O from leaving the catalytic site and reactivate the [Fe-Fe]-hydrogenase H-cluster. In the screening process, polar residues were removed, one at a time, and frequency calculations provided the change in the Gibbs' energy for the dissociation of water (due to their deletion). When residue deletion resulted in significant Gibbs' energy decrease, further residue substitutions have been carried out. Following each substitution, geometry optimization and frequency calculations have been performed to assess the change in the Gibbs' energy for the elimination H₂O. Favorable thermodynamic results have been obtained for both single residue removal ($G_{\text{Glu}^{374}} = -1.6$ kcal/mol), single substitution ($G_{\text{Glu}^{374}\text{His}} = -3.1$ kcal/mol), and combined residue substitutions ($G_{\text{Arg}^{111}\text{Glu;Thr}^{145}\text{Val;Glu}^{374}\text{His;Tyr}^{375}\text{Phe}} = -7.5$ kcal/mol). Because the wild-type enzyme has only an endergonic step to overcome, i.e., for H₂O removal, by eliminating several residues, one at a time, the endergonic step was made to proceed spontaneously. Thus, the most promising residue deletions which enhance H₂O elimination are Arg¹¹¹, Thr¹⁴⁵, Ser¹⁷⁷, Glu²⁴⁰, Glu³⁷⁴, and Tyr³⁷⁵. The thermodynamics and electronic structure analyses show that the bridging carbonyl (CO_b) of the H-cluster plays a concomitant role in the enzyme inhibition/reactivation. In gas phase, CO_b shifts towards Fe_d to compensate for the electron density donated to oxygen upon the elimination of H₂O. However, this is not possible in the wild-type enzyme because the protein matrix hinders the displacement of CO_b towards Fe_d, which leads to enzyme inhibition. However, enzyme reactivation can be achieved by means of appropriate amino acid substitutions.

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Keywords

hydrogenase; H-cluster; density functional theory; quantum mechanics/molecular mechanics calculations; Gibbs' energy; bridging carbonyl; terminal carbonyl; residue substitutions

Introduction

[Fe-Fe]- and [Ni-Fe]-hydrogenases are two major classes of enzymes that reversibly catalyze the apparently simple reaction of protons and electrons to molecular hydrogen, $2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$, which occurs in anaerobic media. In living systems, of the two metalloproteins, [Fe-Fe]-hydrogenases are mostly used for H_2 production, having a reactivity of about 2 orders of magnitude larger than [Ni-Fe]-hydrogenases. These enzymes are found in many bacteria, simple eukaryotes, and archaea where they provide H_2 for the metabolic processes of these life forms. By means of H_2 oxidation, ATP synthesis exploits H_2 as an energy source, whereas H_2 synthesis results from the metabolic disposal of excess electron (with available H^+ s), or from pyruvate fermentation. Proteins, such as ferredoxins, cytochrome C3, and cytochrome C6, act as physiological e^- donors or acceptors⁴⁻⁶. The exploration of alternative energy sources has kindled great interest in hydrogenase research. The reason for studying biological H_2 production is to clarify the complex mechanism (for hydrogen synthesis), which may help researchers produce clean fuel⁷, using certain anaerobic organisms⁸⁻¹¹.

This theoretical study aims to find ways of making these enzymes function aerobically (to provide clean fuel, viz., H_2^{a}), because they become inactivated by exogenous ligands such as O_2 , OH^- , and H_2O ^{1,3}. Water is the metabolic product of the inactivated catalytic site, i.e., $(\text{Fe}_\text{d})\text{OO} \rightarrow (\text{Fe}_\text{d})\text{H}_2\text{O}^1$, and it also binds to the hydrogenase active site in its resting state, viz., $\text{Fe}^{\text{II}}\text{Fe}^{\text{III}}$ ^{12, 13}.

By performing Density Functional Theory (DFT) calculations on the H-cluster, with H_2O , OH^- , and O_2 bound to Fe_d , (redox states $\text{Fe}^{\text{II}}\text{-Fe}^{\text{II}}$), Liu and Hu³ have inferred, based on agreement between calculated and experimental vibrational frequencies of the three endogenous CO ligands, that OH^- is the oxygen species which inhibits hydrogenases. The X-ray structures of [Fe-Fe]-hydrogenases, from *Clostridium pasteurianum* (CPI)¹² and *Desulfovibrio desulfuricans* (DdH)¹⁴, can be used to theoretically investigate their functions via biochemical pathways^{3,15}. Since former DFT, and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations^{1-3,15-22} have shown success in clarifying certain aspects of the catalytic properties of the H-cluster, similar methodologies are also used in our investigation. In our study, as well as in other computational studies^{1-3,15,19,20}, $\text{CH}_3\text{-S}^-$ has been substituted for cysteine, Cys^{382} , and H^+ for the proximal cubane⁶.

The active site of hydrogenases, viz., the H-cluster (Figure 1), is comprised of a dimetal complex, [Fe-Fe], with the metal centers being bridged by di(thiomethyl)amine (DTMA),

^aIn fuel cells, the product, $(\text{H}_2 + \frac{1}{2}\text{O}_2 \rightleftharpoons \text{H}_2\text{O})$, is benign relative to the current, carcinogenic hydrocarbon combustion emissions.
^βA H^+ is replaced for $[\text{Fe}_4\text{-S}_4]^{2+}$, and $\text{CH}_3\text{-S}^-$ for cysteine- S^- in order to minimize computational time, and cost.

and a cubane subunit, $[\text{Fe}_4\text{-S}_4]^{2+}$. The iron atoms are coordinated by endogenous ligands, viz., two cyanides (CN^-), two terminal carbonyls (CO_t), and a bridging carbonyl (CO_b)^γ. An Fe atom, which is part of the proximal cubane subunit, $[\text{Fe}_4\text{-S}_4]^{2+}_p$, is linked to the Fe_p of the di-iron subunit, $[\text{Fe}_p\text{-Fe}_d]$, through a cysteinyl sulfur (or S_γ of Cys³⁸²).

In spite of the di-iron H-cluster subunit redox states, the proximal cubane remains in oxidation²³ state II, $[\text{Fe}_4\text{-S}_4]^{2+}_p$. Computational and experimental [Fe-Fe]-hydrogenase H-cluster (and synthetic H-cluster analogues) research^{1-3,6,7,13-15,19,21,24-52} corroborates the potential redox states of the di-iron H-cluster subunit, $\text{Fe}_p\text{-Fe}_d$, where $\text{Fe}_p^{\text{I}}\text{-Fe}_d^{\text{I}}$, EPR silent, is the reduced di-iron H-cluster subunit, $\text{Fe}_p^{\text{II}}\text{-Fe}_d^{\text{I}}$, paramagnetic, is the partially oxidized, and catalytically active di-iron subunit, and $\text{Fe}_p^{\text{II}}\text{-Fe}_d^{\text{II}}$, EPR silent^{14,53}, is the fully oxidized, inactive biferrous subunit, and has an OH^- or H_2O molecule bound to the Fe_d^{II} .

By performing spectroscopic studies on [Fe-Fe]-hydrogenases, which have been purified from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans*, their catalytic functions have been elucidated^{12-14,54,55}. An X-ray crystal structure of CPI hydrogenase shows an (inactivating) oxygen species that may be OH^- , or H_2O bound to the Fe_d of the H-cluster, while the other X-ray structure has an inactivating CO bound to Fe_d ^{12,13}. For the current study, DdH has been selected because its crystal structure has a better resolution (viz., 1.6 Å), than CPI (viz., 1.8 Å)^{12,14}.

This investigation is subdivided into three parts, viz., thermodynamics, geometric, and electronic analysis, for both wild-type and mutated (residue substituted) DdH. These analyses were carried out in order to understand the thermodynamic results, their relationship to certain molecular spatial behavior, e.g., CO_b movement, and the electronic structural methods, such as frontier molecular orbitals (FMO), and natural bond orbital partial charges (NBO).

Methods

The ONIOM method⁵⁶ (DFT for the QM region, and the universal force field, UFF⁵⁷, for the MM region, implemented in Gaussian03⁵⁸) has been utilized to determine the reaction thermodynamics, viz., ΔG for individual reaction steps, of the [Fe-Fe]-hydrogenase H-cluster (the active group of DdH) reactivation.

Low spin states (singlet, and doublet), and low oxidation states (I, and II) have been used for the di-irons^{3,19} in agreement with experimental and computational data.

The electronic structure of the hydrogenase H-cluster (without proximal cubane) has been determined using DFT method (B3LYP functional^{59,60}), and 6-31+G(d,p) basis set.

Hydrogen atoms were added to the X-ray crystal structure of DdH using the Gromacs program^{61,62} [Brookhaven Protein Data Bank id. 1HFE]. After the structure has been solvated [2043 H_2O molecules (in 1 nm layer peripheral to H-cluster)], six Na^+ ions were

^γThe di-iron atoms are named proximal and distal, $\text{Fe}_p\text{-Fe}_d$ (for Fe_p is closest to the 'proximal' cubane, while Fe_d is 'distal' from the cubane).

randomly incorporated into the solvent (7 Na⁺ ions for clusters 1, 3, 4; 8 Na⁺ ions for cluster 2) to neutralize the negative charges²³ on the H-cluster, medial, and distal cubane/cysteines clusters^δ. At physiological pH (~7), Gromacs program considers negative charges on the acidic residues (28 Asp and 33 Glu), and positive charges on the most basic residues (44 Lys and 15 Arg).

Out of the 13 His found in DdH, only 2 are protonated, and these charges (in conjunction with those from Lys and Arg) are used to neutralize the negatively charged residues, viz., Asp and Glu. Thus, the overall apoprotein charge is zero, except the 12 negatively charged cysteines bound to the iron atoms of the 3 cubanes, where each has a charge of 2+.

Geometry optimizations have been performed in aqueous enzyme phase, where residues in the MM region (except the proximal cubane), and Fe_p and CO_{t,p}, in the QM region have been kept frozen^ε. The rationale for freezing Fe_p and CO_{t,p} arises from former optimizations² where they were found to spatially rearrange the least. Once geometry optimizations have been carried out for DdH, frequency calculations are performed in order to obtain thermodynamic data, viz., G. Frequency calculations treat both the apoenzyme and the cubanes as partial charges, whereas [Fe_p-Fe_d] subunit is treated at DFT level.

The DdH apoenzyme and cubanes, viz., proximal, medial, and distal are included in the MM region. The QM region consists of the [Fe_p-Fe_d] subunit, (the moiety of H-cluster), and C_β, and S_γ (cysteinyl sulfur of Cys³⁸²). In order to avoid dangling bonds between the ONIOM layers, two linking hydrogen atoms were added between S_γ and Fe (of the proximal cubane), and between C_α and C_β (of Cys³⁸²).

The UFF charge equilibration method was utilized to describe the electrostatic interactions within the MM region of DdH, whereas the solvent charges (q_O = -0.706 a.u., and q_H = 0.353 a.u.) were obtained from literature³⁰.

Then, a DdH sphere with a radius of 8 Å from Fe_d was investigated regarding the potential inhibitory residues for H₂O removal. H-cluster hindering residues (for H₂O elimination), are identified by using QM/MM geometry optimizations, and then their influence on thermodynamics, and electronic properties of the catalytic site is assessed. Given that water is polar, candidate, potential inhibitory residues should also be polar. Then, potential, polar inhibitory residues are screened to identify the most probable residues that hinder H₂O from leaving the catalytic site. Screening is the process whereby polar residues are removed (from within a sphere of radius 8 Å), one at a time, which is followed by frequency calculations aiming to learn whether the binding energy of water has decreased. If successful, then further residue substitutions are performed, i.e., a neutral, polar residue is substituted for a neutral, nonpolar residue, and an acidic residue is substituted for a basic residue, and vice versa^ζ. Then, after each substitution, geometry optimization is performed, followed by frequency calculations to obtain the Gibbs' energy of H₂O dissociation.

^δEach of the three cubane/cysteine moieties (found in DdH) is comprised of a cubane plus four surrounding, deprotonated cysteines which are bound to the four iron atoms of every cubane.

^εA 'frozen' enzyme method, (used for hybrid QM/MM setup) has its Cartesian coordinates of the selected atoms kept "fixed" in space, has advantages in acquiring results, and lessens the computational time.

Lastly, Gibbs' energies of H_2O and H_3O^+ (viz., $G_{\text{H}_2\text{O}} = -76.419750$, and $G_{\text{H}_3\text{O}^+} = -76.598767$ Hartrees/molecule), which are needed for thermodynamic analysis, have been obtained by performing frequency calculations on these molecules which were positioned in the H-cluster cavity surrounded by the apoenzyme and the cubanes.

Results

Thermodynamic analysis

The reactivation mechanism of [Fe-Fe]-hydrogenase H-cluster essentially consists of three reaction steps, viz., protonation, reduction, and H_2O elimination. The reactivation pathways (Scheme I, II, and III) proceed with different combinations for the three steps.

For the hybrid calculations, the reductive step, $1 \rightarrow 2$ (Scheme I), of $[\text{Fe}^{\text{II}}\text{-Fe}^{\text{II}}]$ -hydrogenase H-cluster proceeds rather endergonically, viz., $G_{\text{Enzyme } \Phi} = +42.6$ kcal/mol (Table 1) relative to the gas phase ($G_{\text{Gas } \Phi} = +8.2$ kcal/mol, Table 1), which points out that DdH reduction is less spontaneous than in vacuum, emphasizing the stereoelectronic effects of the apoenzyme, the medial, and distal cubanes on the H-cluster. The protonation step, $2 \rightarrow 3$ (Scheme I), is highly exergonic ($G_{\text{Enzyme } \Phi} = -317.9$ kcal/mol, see Supplemental Table 1 for protonation free energies using an alternative protonation source). The Gibbs' energy difference between the reactions of the two environments, (i.e., vacuum vs. enzyme Φ , Table 1), is $+91.3$ kcal/mol, and points to a more spontaneous reaction in gas phase. In step $3 \rightarrow 4$ (H_2O elimination), the hydrogenase H-cluster calculations show a rather small, endergonic result ($G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol), as opposed to the exergonic gas phase outcome ($G_{\text{Gas } \Phi} = -6.6$ kcal/mol). The enzymatic H_2O removal is nonspontaneous due to the influence of the protein environment on the H-cluster electronic properties.

The DdH protonation, $1 \rightarrow 2'$ (Scheme II), provides an exergonic reaction, viz.,

$G_{\text{Enzyme } \Phi} = -236.8$ kcal/mol, proceeding less spontaneously than in gas phase, viz. $G_{\text{Gas } \Phi} = -328.3$ kcal/mol, correlating the trend in Scheme I. The enzyme Φ calculations, for the H_2O removal, $2' \rightarrow 3'$, (Scheme II), confer an endergonic result, ($G_{\text{Enzyme } \Phi} = +22.9$ kcal/mol), as opposed to the exergonic gas Φ reaction step ($G_{\text{Gas } \Phi} = -16.6$ kcal/mol), which results in a difference of $+39.5$ kcal/mol. Again, the effect of the protein environment is manifest on the individual steps of the reaction mechanism. The final reductive step, $3' \rightarrow 4$, of the aqueous enzyme Φ (Scheme II) proceeds exergonically, viz., $G_{\text{Enzyme } \Phi} = -59.4$ kcal/mol, close to the gas phase result, viz., $G_{\text{Gas } \Phi} = -62.7$ kcal/mol.

However, Scheme III, in contrast, definitely provides room for enhancements that could achieve H_2O removal because, in step $3 \rightarrow 4$, the hydrogenase H-cluster calculations provide a rather small, free energy, viz., $G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol, which could be changed into an exergonic reaction via mutagenesis. For Scheme III, only the H_2O removal step is endergonic (viz., $G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol, as in Scheme I), whereas the other remaining steps [protonation (viz., $G_{\text{Enzyme } \Phi} = -236.8$ kcal/mol), reduction (viz., $G_{\text{Enzyme } \Phi} = -38.4$ kcal/mol)] are exergonic.

^ζThe Pymol program⁶³ has been employed to measure interatomic distances between oxygen (of the exogenous H_2O) and terminal atoms (excluding hydrogens) of residue R-groups; it was also used for residue substitutions. The Swiss PDB Viewer⁶⁴ was utilized to add H^+ s to obtain protonated histidines.

The following investigation addresses possible reactivation mechanisms of DdH mutants, uses the QM/MM method for pathway I, II, and III, and aims for the removal of H₂O.

The wild-type hydrogenase has been residue manipulated in two ways. First of all, the considered residues (Table 1) have been removed one at a time in order to find which are responsible for keeping the water from being displaced. Subsequently, for the six culprit residues, that proved to hinder the removal of water, substitutions had been carried out (as describe in the Methods section), viz., Arg¹¹¹Glu, Thr¹⁴⁵Val, Ser¹⁷⁷Ala, Glu²⁴⁰His, Glu³⁷⁴His, and Tyr³⁷⁵Phe.

In Scheme I, the reduction step, 1 → 2, upon the removal of Glu²⁴⁰, becomes less endergonic by $G = +17.0$ kcal/mol (relative to wild-type DdH) but not even close to make it spontaneous[†]. For 2 → 3, with the removal of a basic residue, i.e., Lys²³⁷ (compared to the wild-type enzyme), the protonation step proceeds more exergonically, viz., by a Gibbs' energy difference of +27.3 kcal/mol. The last step, 3 → 4, proceeds exergonically, viz., $G_{\text{Enzyme } \Phi} = -1.6$ kcal/mol, upon the removal of Glu³⁷⁴ from the apoenzyme, however Scheme I cannot proceed to completion due to the endergonic, reductive step, 1 → 2.

For Scheme II, an improvement has been obtained (as in Scheme I) upon the removal of exactly the same residue for each of the corresponding step, i.e., Lys²³⁷ for protonation (viz., $G_{\text{Enzyme } \Phi} = -264.4$ kcal/mol), Glu²⁴⁰ for reduction (viz., $G_{\text{Enzyme } \Phi} = -78.7$ kcal/mol), and Glu³⁷⁴ for H₂O elimination (viz., $G_{\text{Enzyme}} = +21.5$ kcal/mol). However, in spite of Gibbs' energy improvements for all reaction steps, no matter what residues (Table 1) are removed, Scheme II is hindered from completion in the H₂O removal step.

In Scheme III, the only endergonic step is for the removal of H₂O ($G = +2.0$ kcal/mol), which was, nevertheless, made to proceed exergonically by eliminating several residues, one at a time. The most promising residues are Arg¹¹¹, Thr¹⁴⁵, Ser¹⁷⁷, Glu²⁴⁰, Glu³⁷⁴, and Tyr³⁷⁵, and their respective deletion Gibbs' energies are -0.7 kcal/mol, -0.2 kcal/mol, -0.5 kcal/mol, +0.8 kcal/mol, -1.6 kcal/mol, and +1.1 kcal/mol (Table 1). In 3 → 4, single residue substitutions have been carried out on the six residues that hinder the removal of water, viz., Arg¹¹¹Glu, Thr¹⁴⁵Val, Ser¹⁷⁷Ala, Glu²⁴⁰His, Glu³⁷⁴His, and Tyr³⁷⁵Phe, followed by (sequential) frequency calculations.

Then out of the most successful substitutions, two, three, and four residue combinations were examined by frequency calculations (Table 2). The 1st two-residue combination is Glu³⁷⁴His, and Tyr³⁷⁵Phe giving $G = -5.1$ kcal/mol; the 2nd three-residue combination is Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe giving $G = -6.2$ kcal/mol, and the 3rd four-residue combination is Arg¹¹¹Glu, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe giving $G = -7.5$ kcal/mol.

Geometric Considerations

In order to explain Gibbs' energies between the aqueous enzyme Φ and the gas Φ calculations, DdH H-cluster and H-cluster geometries for the two phases are analyzed, and

[†]To improve the electron transfer step, given that a negative residue (such as Glu²⁴⁰) has been removed, one should proceed towards the positively charged residues, i.e., basic residues.

then the hydrogenase H-cluster distances to the six replaced, juxtaposed residues are presented for the aqueous enzyme Φ .

The wild-type DdH QM/MM calculations for H₂O removal, (3 $\rightarrow$ 4), reveal a contrasting picture regarding CO_b translation towards Fe_d relative to the gas phase H₂O elimination. That is, in Table 3⁰, 3 $\rightarrow$ 4, the iron-carbon distance, Fe_d-CO_b, essentially remains constant [(viz., 1.907 Å $\rightarrow$ 1.908 Å), whereas the gas Φ distance becomes smaller (viz., 1.945 Å $\rightarrow$ 1.850 Å), and the reaction (3 $\rightarrow$ 4) is exergonic]^{2,15}, which may explain why H₂O removal is endergonic for the enzyme Φ .

Thus, it is ascertained that for the aqueous enzyme Φ , an endergonic ligand (H₂O) dissociation is manifested in the Fe_p-CO_b bond contraction (1.959 Å $\rightarrow$ 1.939 Å). The opposite trend is observed in gas phase, where the Fe_p-CO_b bond is elongated (2.013 Å $\rightarrow$ 2.232 Å) after H₂O elimination.

Out of the three reaction pathways presented here, only Scheme III is analyzed for geometrical considerations, for it is the only one having the potential for metabolic reactivation by means of residue substitutions. The latter scheme has only the water removal step to be overcome in order to proceed exergonically, while, on the other hand, the other schemes cannot be made exergonic by residue substitutions.

Next, an analysis is provided for the interatomic distances (Å), between Fe_p and Fe_d, Fe_p and CO_b, Fe_d and CO_b, and Fe_d and H₂O, before and after water removal, to compare the H₂O elimination Gibbs' energies for the residue substituted DdH with the thermodynamics of the wild-type DdH.

For removal of water from the wild-type enzyme, the distance between Fe_d and CO_b is slightly increasing (Table 3), which corresponds to the endergonic step, viz., $G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol. However, the H₂O removal from the mutated enzyme is exergonic (Table 2), correlating to the movement of the CO_b towards the Fe_d. As a result of mutating DdH, the following bond contractions, Fe_d-CO_b, have been obtained, viz., 0.024 Å for Arg¹¹¹Glu, 0.028 Å for Thr¹⁴⁵Val, 0.017 Å for Glu³⁷⁴His, and 0.031 Å for Tyr³⁷⁵Phe, corresponding to exergonic steps for water elimination (Table 2).

Note that a simultaneous bond elongation occurs [for all presented mutations (Table 3)] between the Fe_p and the bridging carbonyl, Fe_p-CO_b, when bond contraction for Fe_d-CO_b takes place. As a result of mutating DdH (vs. the wild-type enzyme), larger bond contractions between the iron atoms, Fe_d-Fe_p, have been obtained (ca. 0.1 Å, Table 3). It is also noticed that the bond length between the distal iron and water, Fe_d-H₂O, is longer, i.e., about 2.2 Å (vs. 2.1 Å) in the mutated DdH vs. the wild-type enzyme.

The following trend has been observed for some of the DdH mutants that the closer the substituted residue is to the H-cluster exogenous water, the more spontaneous the water removal step becomes. The exception is Tyr³⁷⁵Phe, in which the substituted amino acid is

⁰In gas Φ , both Scheme I and II for H₂O removal show a concerted bond elongation between Fe_p-CO_b, and a bond contraction between Fe_d-CO_b, as seen in 3 $\rightarrow$ 4, and 2' $\rightarrow$ 3' for Schemes 1, and 2, respectively. Hence, the shifting of the CO_b towards Fe_d seems to facilitate the exogenous ligand removal, e.g., H₂O, and H₂.

highly hydrophobic, although it gets closest to the exogenous oxygen atom of H₂O, it nevertheless has less effect on removing water compared to Glu³⁷⁴His mutant where the substituted amino acid (at a greater distance from H₂O) is not only polar but of opposite charge as well.

The negative partial charge on oxygen (-0.935) of H₂O repels the negatively charged carboxylate of Glu³⁷⁴ ($q_{\text{COO}^-} = 0.547, -0.567, -0.563$), thus making the water removal difficult. When Glu³⁷⁴ is replaced by a protonated histidine, the opposite effect is observed.

Frontier Molecular Orbital Analysis

Molecular orbital analysis is provided using frontier orbitals (HOMO, LUMO, and SOMO) in correlation with the formerly presented Gibbs' energies. Essentially, reduction for all three pathways are carried out on closed-shell clusters, that is, an e⁻ is transferred into the lowest unoccupied molecular orbital, H-cluster LUMO.

In the case of open-shell H-cluster protonation (both gas and aqueous enzyme Φ), a σ -bond is formed between a H⁺ and (the exogenous ligand) OH⁻ by the interaction of electrons in the highest occupied molecular orbitals, viz., SOMO, and HOMO. Conversely, when a H⁺ is in close proximity to a closed-shell H-cluster, the resulting σ -bond is mainly due to the contribution of e⁻s from HOMO and the H⁺.

The [Fe-Fe]-hydrogenase H-cluster 1 of the wild-type enzyme becomes reduced (Scheme I, 1 $\rightarrow$ 2), and according to the LUMO depiction (Figure 2), the transferred e⁻ appears to be localized in the vicinity of the di-iron atoms, Fe_p-Fe_d. However, according to the NBO partial charge results (Figure 3), the iron atoms (of hydrogenase H-cluster 1) do not have affinity for the approaching e⁻, i.e., $q_{\text{Fe}_p} = -1.291$ a.u., and $q_{\text{Fe}_d} = -1.109$ a.u. The fact that the reduction is endergonic ($G_{\text{Enzyme } \Phi} = +42.6$ kcal/mol) corroborates the orbital analysis results, which indicate that e⁻ transfer to the cluster should be thermodynamically unfavorable due to existing negative charge on the di-iron atoms.

Regarding [Fe-Fe]-hydrogenase H-cluster 2, (2 $\rightarrow$ 3), the open shells, SOMO _{α} , HOMO _{α} , and HOMO _{β} , have similar orbital distribution over OH⁻ (Figure 2), and could make a σ -bond with the incoming H⁺; in conjunction with the NBO partial charge of the hydroxyl oxygen, $q_{\text{O}} = -0.991$ a.u., the large H⁺ affinity for cluster 2, $G = -317.9$ kcal/mol, (2 $\rightarrow$ 3) comes as no surprise.

In H-cluster 3, (3 $\rightarrow$ 4), the open shells, HOMO _{α} , HOMO _{β} , and SOMO _{α} , are diffused throughout the cluster except over the Fe_d-OH₂ bond, implying that the e⁻s of the σ -bond, Fe_d-OH₂, reside in a lower energy state, which also explains the negative NBO partial charges of both Fe_d ($q_{\text{Fe}_d} = -1.012$ a.u.), and the O ($q_{\text{O}} = -0.935$ a.u.) of H₂O. This fact may explain, therefore, the affinity of Fe_d for H₂O, i.e., $G = +2.0$ kcal/mol.

For [Fe-Fe]-hydrogenase H-cluster 1, (Scheme II, 1 $\rightarrow$ 2'), the HOMO is more diffused over Fe_d, DTMA bidentate ligand, and over the exogenous ligand, i.e., OH⁻. In spite of greater e⁻ orbital diffusion over the DTMA ligand, the H⁺ becomes captured by OH⁻, for it is a stronger base than the N of the DTMA bridge, as the NBO charges indicate ($q_{\text{O}} = -0.898$;

$q_N = -0.682$). Note that these analyses also agree with the calculated hydrogenase H-cluster proton affinity, i.e., $G = -236.8$ kcal/mol.

For H-cluster 2', (Scheme II, $2' \rightarrow 3'$), HOMO is diffused over the center of Fe_p - Fe_d subcluster, CO_b , and over CN^- (Fe_d coordinated) which means that the e^- s of the σ -bond, Fe_d - OH_2 , are situated in a lower energy state, explaining the negative NBO partial charges of both O (of H_2O , $q_O = -0.878$ a.u.), and Fe_d ($q_{Fe_d} = -1.077$ a.u.). This, then, accounts for the bond strength of Fe_d for H_2O , i.e., $G = +22.9$ kcal/mol. In addition, from geometrical considerations, $2' \rightarrow 3'$, it can be seen that CO_b - Fe_d bond becomes longer, viz., $1.932 \rightarrow 1.942$ Å [vs. $1.907 \rightarrow 1.908$ Å (in H-cluster 3, Scheme I, $3 \rightarrow 4$)], which partially accounts for the more endergonic H_2O elimination process, coinciding with an increase of one order of magnitude for the Gibbs' energy difference (for water elimination in $2' \rightarrow 3'$ vs. $3 \rightarrow 4$).

In H-cluster 3', LUMO is mostly localized on the potential catalytic binding site. Upon the reduction of the H-cluster, ($3' \rightarrow 4$), the LUMO depiction shows that the e^- should become localized peripherally to the Fe_d . Then according to the NBO partial charges, i.e., $q_{Fe_p} = -1.317$ a.u., and $q_{Fe_d} = -0.733$ a.u., in conjunction with the above presented LUMO depiction, the cluster reduction occurs with a rather high spontaneity, i.e., $G_{Enzyme \Phi} = -59.4$ kcal/mol, although the di-iron atoms have rather high NBO charges^l.

The LUMO on the wild-type hydrogenase H-cluster 2', is delocalized on the two irons, Fe_p - Fe_d , DTMA sulfur atoms, and CO_b (Figure 2). Cluster reduction (Scheme III, $2' \rightarrow 3$) occurs with a rather high spontaneity, i.e., $G_{Enzyme \Phi} = -38.4$ kcal/mol, although the di-iron atoms have high NBO negative charges, i.e., $q_{Fe_p} = -1.305$ a.u., and $q_{Fe_d} = -1.077$ a.u. [just as for H-cluster 3', ($3' \rightarrow 4$)]. It ought to be noted that the e^- transfer occurs exergonically relative to the endergonic step (+42.6 kcal/mol) in Scheme I, $1 \rightarrow 2$, perhaps because the total charge on each cluster is different, i.e., cluster 2' has charge 0 a.u., while 1 has a charge of -1 a.u.

Discussion

The comparison between gas phase and wild-type enzyme calculations, presented in the current study, unambiguously shows that CO_b migration is key to enzyme inhibition by O_2 . When CO_b is close to the Fe_d , DdH becomes reactivated, while, on the other hand, when CO_b is further away from Fe_d , wild-type enzyme H-cluster inactivation is observed. The displacement of CO_b is controlled by the apoenzyme, and can be further modulated by amino acid substitutions. In the wild-type enzyme, the protein environment impinges CO_b away from the catalytic site, Fe_d , leading to an exogenously inhibited hydrogenase, and thus hindering H_2O elimination. On the contrary, suitable residue substitutions can reverse the enzyme inhibition by allowing CO_b to migrate towards Fe_d , concomitant with H_2O removal.

The potential reactivation pathway of Scheme I is rendered less promising by the protein environment due to step $1 \rightarrow 2$, which is rather endergonic.

^lBecause NBO charges on iron atoms are both negatively charged, and the reaction proceeds exergonically, it means that the e^- is transferred due to a vicinal potential difference, with the e^- source most likely being the proximal cubane/cysteine (Fe_4S_4/Cys_4), cluster.

Also, the reactivation pathway of [Fe-Fe]-hydrogenase H-cluster 4 (Scheme II) cannot be realized because of the high nonspontaneous step for H₂O removal ($2' \rightarrow 3'$, $G_{\text{Enzyme } \Phi} = +22.9 \text{ kcal/mol}$). As a result, this scheme does not seem to provide room for mutagenic enhancements (residue substitutions) that could improve H₂O removal.

However, in electrochemical settings, a hydrogenase can be adsorbed onto an electrode surface where the endergonic reductive step may be reversed by intramolecular changes (viz., apoenzyme amino acid substitutions), or extramolecular modifications (viz., voltage adjustment, or solution tuning such as pH modification, salt concentration changes, etc.).

The last reaction pathway of Scheme III provides the best chance for reactivating DdH, because the only reaction step to overcome (H₂O removal) is barely endergonic ($G_{\text{Enzyme } \Phi} = +2.0 \text{ kcal/mol}$), which can be accomplished by suitable amino acid substitutions.

DdH screening by residue deletions pointed out which residues may increase the removal of H₂O from the catalytic site. From residue deletion clues, single and multiple residue substitutions have led to exergonic H₂O removal (e.g., Arg¹¹¹Glu, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe providing $G = -7.5 \text{ kcal/mol}$).

Conclusions

The DdH reactivation, according to pathway I, consists of an endergonic e^- transfer step (viz., $G_{\text{Enzyme } \Phi} = +42.6 \text{ kcal/mol}$), followed by an exergonic H^+ transfer step (viz., $G_{\text{Enzyme } \Phi} = -317.9 \text{ kcal/mol}$), and then an endergonic H₂O removal step (viz., $G_{\text{Enzyme } \Phi} = +2.0 \text{ kcal/mol}$).

For reactivation pathway II, the [Fe-Fe]-hydrogenase H-cluster H^+ transfer occurs first ($G_{\text{Enzyme } \Phi} = -236.8 \text{ kcal/mol}$), followed by H₂O removal ($G_{\text{Enzyme } \Phi} = +22.9 \text{ kcal/mol}$), and then by e^- transfer ($G_{\text{Enzyme } \Phi} = -59.4 \text{ kcal/mol}$), with all steps being exergonic, except for the removal of water.

Pathway III, however, proceeds by an exergonic protonation step (viz., $G_{\text{Enzyme } \Phi} = -236.8 \text{ kcal/mol}$), an exergonic e^- transfer step (viz., $G_{\text{Enzyme } \Phi} = -38.4 \text{ kcal/mol}$), followed by water removal step (viz., $G_{\text{Enzyme } \Phi} = +2.0 \text{ kcal/mol}$), which is barely endergonic. Thus, the reason DdH intermediates of pathway III, rather than those of pathway I and II, were chosen to be residue mutated was to achieve a pathway that proceeds exergonically throughout. Results have been obtained for deleted, substituted, and combined (substitutions of) residues. Combinations of two, three, and four residues gave improved negative Gibbs' energies for the removal of water, relative to single substituted residues.

In pathway III, the endergonic step, for H₂O removal, was made to proceed more spontaneously by removing inhibitory residues, one at a time. The promising residues are Arg¹¹¹, Thr¹⁴⁵, Ser¹⁷⁷, Glu²⁴⁰, Glu³⁷⁴, and Tyr³⁷⁵, and their respective Gibbs' energies are -0.7 kcal/mol, -0.2 kcal/mol, -0.5 kcal/mol, +0.8 kcal/mol, -1.6 kcal/mol, and +1.1 kcal/mol.

Individual residues were substituted, viz., Arg¹¹¹Glu, Thr¹⁴⁵Val, Ser¹⁷⁷Ala, Glu²⁴⁰His, Glu³⁷⁴His, and Tyr³⁷⁵Phe. All substitutions resulted in improved spontaneity for H₂O removal, relative to residue deletions, except for Ser¹⁷⁷ → Ser¹⁷⁷Ala ($G = -0.5$ kcal/mol → $G = -0.1$ kcal/mol), and Glu²⁴⁰ → Glu²⁴⁰His ($G = +0.8$ kcal/mol → $G = +1.1$ kcal/mol).

From the successful, single residue substitutions, two, three, and four residue combinations were used to prepare DdH mutants for frequency calculations. The two residue combination, Glu³⁷⁴His, and Tyr³⁷⁵Phe, resulted in $G = -5.1$ kcal/mol; the three residue combination, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe, provided a $G = -6.2$ kcal/mol, and the four residue combination, Arg¹¹¹Glu, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe, gave a $G = -7.5$ kcal/mol. Though the H₂O removal thermodynamic trend is not precisely cumulative, it seems to point in that direction.

The wild-type DdH H-cluster bond distance, i.e., Fe_d-CO_b, relative to that of the H-cluster (gas Φ), for H₂O elimination remains almost constant (viz., 1.907 Å → 1.908 Å), whereas the gas Φ^2 bond distance becomes smaller (viz., 1.945 Å → 1.850 Å), occurring with a concomitant exergonic H₂O removal; this, then, may partly explain why the removal of H₂O is exergonic for gas Φ , as opposed to the enzyme Φ .

Since pathway III has only the H₂O removal step to overcome to proceed exergonically, it was found to provide, to some extent, the sought after reactivation via residue substitutions. The step for H₂O removal from the wild-type hydrogenase ($G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol) shows that the distance between the Fe_d and CO_b remains approximately the same. However, the H₂O removal from the mutated enzyme proceeds exergonically, correlating to the movement of the CO_b towards the Fe_d. The following bond contractions, Fe_d-CO_b, have been obtained, viz., 0.024 Å for Arg¹¹¹Glu, 0.028 Å for Thr¹⁴⁵Val, 0.017 Å for Glu³⁷⁴His, and 0.031 Å for Tyr³⁷⁵Phe, corresponding to exergonic steps for water elimination. Additionally, bond contractions have been obtained between the iron atoms (ca. 0.1 Å), concurrent with H₂O dissociation.

We conclude by postulating that even a single and proper residue (experimental) substitution, like Glu³⁷⁴ → Glu³⁷⁴His ($G = -1.6$ kcal/mol → $G = -3.1$ kcal/mol), can reactivate the [Fe-Fe]-hydrogenase.

We presented here a complete mechanistic picture of the reactivation reaction of the H-cluster and the role of the bridging carbonyl in modulating this reactivation. We compared the gas phase results with enzyme calculations and show how careful residue mutations can have a significant effect on the electronic structure of H-cluster. We have identified for the first time amino acid residues surrounding the active site that can be used to modulate the reactivity of [Fe-Fe]-hydrogenase. Our calculations and the different analyses (i.e., thermodynamic, geometrical, and electronic) emphasize that all these structural data pertaining to different origins (electronic, geometric, thermodynamics) concur in the suggested picture for the reaction mechanism of the reactivation of wild-type [Fe-Fe]-hydrogenase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Dogaru D, Motiu S, Gogonea V. *Int J Quantum Chem.* 2008; 108 online.
2. Motiu S, Dogaru D, Gogonea V. *Int J Quantum Chem.* 2007; 107:1248–1252.
3. Liu Z-P, Hu P. *J Am Chem Soc.* 2002; 124:5175–5182. [PubMed: 11982382]
4. Vignais PM, Billoud B, Meyer J. *FEMS Microbiol Rev.* 2001; 25:455–501. [PubMed: 11524134]
5. Adams MWW, Stiefel EI. *Science.* 1998; 282:1842–1843. [PubMed: 9874636]
6. Frey M. *Chembiochem.* 2002; 3:153–160. [PubMed: 11921392]
7. Liu X, Ibrahim SK, Tard C, Pickett CJ. *Coord Chem Rev.* 2005; 15-16:1641–1652.
8. Adams MWW. *Biochim Biophys Acta.* 1990; 1020:115–145. [PubMed: 2173950]
9. Albracht SPJ. *Biochim Biophys Acta.* 1994; 1118:167–204. [PubMed: 7803444]
10. Happe RP, Roseboom W, Pierik AJ, Albracht SP, Bagley KA. *Nature.* 1997; 385:126. [PubMed: 8990114]
11. Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. *Plant Physiol.* 2000; 122:127–135. [PubMed: 10631256]
12. Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC. *Science.* 1998; 282:1853–1858. [PubMed: 9836629]
13. Lemon BJ, Peters JW. *Biochemistry.* 1998; 38:12969–12973. [PubMed: 10529166]
14. Nicolet Y, Piras C, Legrand P, Hatchikian EC, Fontecilla-Camps JC. *Structure.* 1999; 7:13–23. [PubMed: 10368269]
15. Liu Z-P, Hu P. *J Chem Phys.* 2002; 117:8177–8180.
16. Bruschi M, Fantucci P, De Gioia L. *Inorg Chem.* 2002; 41:1421–1429. [PubMed: 11896710]
17. Bruschi M, Fantucci P, De Gioia L. *Inorg Chem.* 2003; 42:4773–4781. [PubMed: 12870970]
18. Bruschi M, Fantucci P, De Gioia L. *Inorg Chem.* 2004; 43:3733–3741. [PubMed: 15180430]
19. Cao Z, Hall MB. *J Am Chem Soc.* 2001; 123:3734–3742. [PubMed: 11457105]
20. Fan H-J, Hall MB. *J Am Chem Soc.* 2001; 123:3828–3829. [PubMed: 11457119]
21. Greco C, Bruschi M, De Gioia L, Ryde U. *Inorg Chem.* 2007; 46:5911–5921. [PubMed: 17602468]
22. Zampella G, Bruschi M, Fantucci P, Razavet M, Pickett CJ, De Gioia L. *Chem Eur J.* 2005; 11:509–520. [PubMed: 15578644]
23. Popescu CV, Munck E. *J Am Chem Soc.* 1999; 121:7877–7884.
24. Tye JW, Darensbourg MY, Hall MB. *J Comput Chem.* 2006; 27:1454–1462. [PubMed: 16807976]
25. Darensbourg MY. *Nature.* 2005; 433:589–591. [PubMed: 15703733]
26. Madamwar D, Garg N, Shah V. *World J Microbiol Biotechnol.* 2000; 16:757–767.
27. He CJ, Wang M, Li MA, Sun LC. *Prog Chem.* 2004; 16:250–255.
28. Chang CH, King PW, Ghirardi ML, Kim K. *Biophys J.* 2007; 93:3034–3045. [PubMed: 17660315]
29. Long H, Chang CH, King PW, Ghirardi ML, Kim K. *Biophys J.* 2008; 95:3753–3766. [PubMed: 18621810]
30. Rappe AK, Goddard WA. *J Phys Chem.* 1991; 95:3358–3363.

31. Zilberman S, Stiefel EI, Cohen MH, Car R. *Inorg Chem.* 2006; 45:5715–5717. [PubMed: 16841968]
32. Silaghi-Dumitrescu I, Bitterwolf TE, King RB. *J Am Chem Soc.* 2006; 128:5342–5343. [PubMed: 16620096]
33. Boyke CA, Rauchfuss TB, Wilson SR, Rohmer M-M, Benard M. *J Am Chem Soc.* 2004; 126:15151–15160. [PubMed: 15548012]
34. Adams MWW, Mortenson LE. *J Biol Chem.* 1984; 259:7045–7055. [PubMed: 6327705]
35. Rusnak FM, Adams MWW, Mortenson LE, Munck E. *J Biol Chem.* 1987; 262:38–41. [PubMed: 3025213]
36. Adams MWW. *J Biol Chem.* 1987; 262:15054–15061. [PubMed: 2822711]
37. Trohalaki S, Pachter R. *ENERG FUEL.* 2007; 21:2278–2286.
38. Greco C, Bruschi M, Heimdal J, Fantucci P, De Gioia L, Ryde U. *Inorg Chem.* 2007; 46:7256–7258. [PubMed: 17676838]
39. Greco C, Bruschi M, Fantucci P, De Gioia L. *Eur J Inorg Chem.* 2007; 13:1835–1843.
40. Greco C, Zampella G, Bertini L, Bruschi M, Fantucci P, De Gioia L. *Inorg Chem.* 2007; 46:108–116. [PubMed: 17198418]
41. Bruschi M, Zampella G, Fantucci P, De Gioia L. *Coord Chem Rev.* 2005; 15-16:1620–1640.
42. Armstrong FA. *Curr Opin Chem Biol.* 2004; 8:133–140. [PubMed: 15062773]
43. Rauchfuss TB. *Inorg Chem.* 2004; 43:14–26. [PubMed: 14704049]
44. Evans DJ, Pickett CJ. *Chem Soc Rev.* 2003; 35:268–275. [PubMed: 14518180]
45. Chen Z, Lemon BJ, Huang S, Swartz DJ, Peters JW, Bagley KA. *Biochemistry.* 2002; 41:2036–2043. [PubMed: 11827551]
46. Horner DS, Heil B, Happe T, Embley TM. *Trends Biochem Sci.* 2002; 27:148–153. [PubMed: 11893512]
47. Nicolet Y, Cavazza C, Fontecilla-Camps JC. *J Inorg Biochem.* 2002; 91:1–8. [PubMed: 12121756]
48. Lyon EJ, Georgakaki IP, Reibenspies JH, Darensbourg MY. *Angew Chem Int Ed.* 1999; 38:3178–3180.
49. Cloirec AL, Best SP, Borg S, Davies SC, Evans DJ, Hughes DL, Pickett CJ. *Chem Commun.* 1999:2285–2286.
50. Rauchfuss TB, Contakes SM, Schmidt M. *J Am Chem Soc.* 1999; 121:9736–9737.
51. Lai C-H, Lee W-Z, Miller ML, Reibenspies JH, Darensbourg DJ, Darensbourg MY. *J Am Chem Soc.* 1998; 120:10103–10114.
52. Zambrano IC, Kowal AT, Mortenson LE, Adams MWW, Johnson MK. *J Biol Chem.* 1989; 264:20974–20983. [PubMed: 2556390]
53. Pierik AJ, Hagen WR, Redeker JS, Wolbert RBG, Boersma M, Verhagen MF, Grande HJ, Veeger C, Mustsaers PHA, Sand RH, Dunham WR. *Eur J Biochem.* 1992; 209:63–72. [PubMed: 1396719]
54. De Lacey AL, Stadler C, Cavazza C, Hatchikian EC, Fernandez VM. *J Am Chem Soc.* 2000; 122:11232–11233.
55. Nicolet Y, De Lacey AL, Vernede X, Fernandez VM, Hatchikian EC, Fontecilla-Camps JC. *J Am Chem Soc.* 2001; 123:1596–1601. [PubMed: 11456758]
56. Dapprich S, Komaromi I, Byun KS, Morokuma K, Frisch MJ. *J Mol Struct (Theochem).* 1999; 461:1–21.
57. Rappe AK, Casewit CJ, Colwell KS, Goddard WA, Skiff WM. *UFF. J Am Chem Soc.* 1992; 113:10024–10035.
58. Frisch, MJ.; Trucks, GW.; Schlegel, HB.; Scuseria, GE.; Robb, MA.; Cheeseman, JR.; Montgomery, JA., J.; Vreven, T.; Kudin, KN.; Burant, JC.; Millam, JM.; Iyengar, SS.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, GA.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, JE.; Hratchian, HP.; Cross, JB.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, RE.; Yazyev, O.; Austin, AJ.; Cammi, R.; Pomelli, C.; Ochterski, JW.; Ayala, PY.; Morokuma, K.; Voth, GA.; Salvador, P.; Dannenberg, JJ.;

Zakrzewski, VG.; Dapprich, SD.; D, A.; Strain, MC.; Farkas, O.; Malick, DK.; Rabuck, AD.; Raghavachari, K.; Foresman, JB.; Ortiz, JV.; Cui, Q.; Baboul, AG.; Clifford, S.; Cioslowski, J.; Stefanov, BB.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, RL.; Fox, DJ.; Keith, T.; Al-Laham, MA.; Peng, CY.; Nanayakkara, A.; Challacombe, M.; Gill, PMW.; Johnson, B.; Chen, W.; Wong, MW.; Gonzalez, C.; Pople, JA. Gaussian 03, Revision C.02. Gaussian, Inc.; Wallingford CT: 2004.

59. Becke AD. J Chem Phys. 1993; 98:5648–5652.
60. Lee C, Yang W, Parr RG. Phys Rev B. 1988; 37:785–789.
61. Berendsen HJC, van der Spoel D. Comp Phys Comm. 1995; 91:43–56.
62. Lindahl E, Hess B. J Mol Mod. 2001; 7:306–317.
63. DeLano, WL. The PyMOL Molecular Graphics System. DeLano Scientific; Palo Alto, CA, USA: 2002.
64. Guex N, Peitsch MC. Electrophoresis. 1997; 18:2714–2723. [PubMed: 9504803]

Figure 1.
The H-cluster Structure.

Figure 2.
The Frontier Molecular Orbitals from DFT Calculations (B3LYP/6-31+G(d,p)).

Figure 3. The NBO Charges Obtained from DFT Calculations (B3LYP/6-31+G(d,p)). Charges are Given in a.u. for the Following H-Clusters: 1, 2, 2', 3, 3', and 4 Starting from the Top of the Columns.

H-cluster Reactivation

Scheme I.

The Reactivation Pathway I of [Fe-Fe]-hydrogenase H-cluster.

H-cluster Reactivation

Scheme II.

The Reactivation Pathway II of [Fe-Fe]-hydrogenase H-cluster.

H-cluster Reactivation

Scheme III.

The Reactivation Pathway III of [Fe-Fe]-hydrogenase H-cluster.

Table 1

Native and residue removed DdH Gibbs' energies (kcal/mol) for reaction steps of reactivation pathways I, II, and III.

Reaction steps	(+e ⁻) 1 → 2	(+H ⁺) 2 → 3	(-H ₂ O) 3 → 4	(+H ⁺) 1 → 2'	(-H ₂ O) 2' → 3'	(+e ⁻) 3' → 4	(+e ⁻) 2' → 3
Native DdH	+42.6	-317.9	+2.0	-236.8	+22.9	-59.4	-38.4
Ser ⁶² *	+38.3	-313.6	+2.0	-232.6	+23.1	-63.9	-42.7
Arg ¹¹¹	+59.0	-332.4	-0.7	-251.4	+22.0	-44.7	-22.0
Tyr ¹¹²	+47.5	-322.1	+1.6	-241.1	+22.8	-54.8	-33.6
Asp ¹⁴⁴	+28.8	-305.0	+3.0	-223.4	+24.1	-73.8	-52.8
Thr ¹⁴⁵	+44.1	-318.8	-0.2	-237.9	+22.8	-59.9	-36.9
Glu ¹⁴⁶	+32.5	-308.5	+2.5	-227.4	+23.6	-69.7	-48.6
Thr ¹⁴⁸	+41.8	-316.8	+1.8	-235.8	+22.9	-60.3	-39.2
Asp ¹⁵⁰	+32.8	-308.1	+2.1	-227.0	+23.3	-69.5	-48.3
Thr ¹⁵²	+47.1	-322.6	+1.9	-241.6	+23.0	-55.1	-33.9
Glu ¹⁵⁵	+36.5	-311.4	+1.9	-230.2	+23.0	-65.8	-44.7
Thr ¹⁷⁶	+43.8	-319.5	+2.1	-238.3	+23.2	-58.5	-37.4
Ser ¹⁷⁷	+38.3	-312.8	-0.5	-231.8	+22.9	-66.1	-42.7
Gln ¹⁸³	+46.3	-322.3	+2.3	-241.1	+23.3	-55.9	-34.9
Ser ¹⁹⁸	+42.6	-318.0	+2.1	-236.9	+23.2	-59.6	-38.5
Lys ²⁰¹	+48.3	-323.5	+1.7	-242.6	+22.7	-53.6	-32.6
Ser ²⁰²	+46.3	-324.1	+2.4	-241.8	+22.5	-56.0	-35.9
Asn ²⁰⁷	+46.7	-321.6	+1.5	-240.6	+22.5	-55.2	-34.2
Ser ²³⁰	+38.2	-314.0	+2.3	-232.9	+23.3	-64.0	-42.9
Lys ²³⁷	+69.2	-345.2	+1.2	-264.4	+21.7	-32.1	-11.6
Lys ²³⁸	+47.4	-322.1	+1.7	-241.5	+22.9	-54.4	-33.3
Glu ²⁴⁰	+25.6	-300.1	+0.8	-218.9	+23.8	-78.7	-55.6
Thr ²⁵⁷	+42.8	-318.0	+1.9	-236.9	+23.1	-59.5	-38.3
Thr ²⁵⁹	+45.9	-320.8	+1.8	-239.9	+23.0	-56.1	-35.0
Thr ²⁶⁰	+42.1	-317.4	+2.0	-236.4	+23.1	-60.1	-39.0
Ser ²⁸⁹	+44.0	-319.1	+1.9	-238.1	+22.9	-58.1	-37.0

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Reaction steps	(+e ⁻) 1 → 2	(+H ⁺) 2 → 3	(-H ₂ O) 3 → 4	(+H ⁺) 1 → 2 [*]	(-H ₂ O) 2 [*] → 3 [*]	(+e ⁻) 3 [*] → 4	(+e ⁻) 2 [*] → 3
Thr ²⁹⁴	+40.4	-316.0	+2.2	-234.9	+23.2	-61.8	-40.8
Thr ²⁹⁹	+40.9	-316.2	+1.9	-235.1	+23.0	-61.2	-40.1
Glu ³⁷⁴	+27.7	-301.3	-1.6	-220.2	+21.5	-76.5	-53.4
Tyr ³⁷⁵	+41.5	-315.8	+1.1	-234.8	+22.3	-60.6	-39.5
Gln ³⁸⁸	+44.0	-319.2	+2.0	-238.1	+23.1	-58.2	-37.1
Gas φ	+8.2	-409.2	-6.6	-328.3	-16.6	-62.7	-72.7

* Residue removed DdH;
s = small chain

Table 2

DdH Gibbs' energies (kcal/mol) of one, two, three, and four residue mutations for reaction step (3 $\rightarrow$ 4).

Residue substitutions for H ₂ O removal step	3 $\rightarrow$ 4
Arg ¹¹¹ Glu	-0.9
Thr ¹⁴⁵ Val	-1.3
Ser ¹⁷⁷ Ala	-0.1
Glu ²⁴⁰ His	+1.1
Glu ³⁷⁴ His	-3.1
Tyr ³⁷⁵ Phe	-2.1
Combinations of:	
Glu ³⁷⁴ His and Tyr ³⁷⁵ Phe	-5.1
Thr ¹⁴⁵ Val, Glu ³⁷⁴ His and Tyr ³⁷⁵ Phe	-6.2
Arg ¹¹¹ Glu, Thr ¹⁴⁵ Val, Glu ³⁷⁴ His and Tyr ³⁷⁵ Phe	-7.5

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Table 3

Interatomic distances (Å) for wild-type and mutated DdH, between Fe_p and Fe_d, Fe_p and CO_b, Fe_d and CO_b, and Fe_d and H₂O, before and after H₂O removal (3 → 4).

Before H ₂ O removal	3 (Wild-type)	3 (Arg ¹¹¹ Glu mutant)	3 (Thr ¹⁴⁵ Val mutant)	3 (Glu ³⁷⁴ His mutant)	3 (Tyr ³⁷⁵ Phe mutant)
Fe _p -Fe _d	2.626	2.659	2.670	2.690	2.666
CO _b -Fe _p	1.959	1.963	1.968	1.975	1.991
CO _b -Fe _d	1.907	1.952	1.963	1.945	1.937
Fe _d -O(H ₂ O)	2.127	2.205	2.181	2.223	2.184
After H ₂ O removal	4 (Wild-type)	4 (Arg ¹¹¹ Glu mutant)	4 (Thr ¹⁴⁵ Val mutant)	4 (Glu ³⁷⁴ His mutant)	4 (Tyr ³⁷⁵ Phe mutant)
Fe _p -Fe _d	2.587	2.584	2.616	2.589	2.582
CO _b -Fe _p	1.939	2.006	2.003	2.008	2.005
CO _b -Fe _d	1.908	1.928	1.935	1.928	1.906

Table 4

Interatomic distances between the oxygen (of exogenous H₂O; Fe_d-OH₂, compound **3**) and the juxtaposed atoms of the residue R-groups.

Mutated DdH	H ₂ O oxygen	Å
Arg¹¹¹Glu mutant		
O(ε)	O(H ₂ O)	17.507
O(ε)	O(H ₂ O)	18.259
Thr¹⁴⁵Val mutant		
C(γ)	O(H ₂ O)	9.616
C(γ)	O(H ₂ O)	9.650
Ser¹⁷⁷Ala mutant		
C(β)	O(H ₂ O)	6.077
Glu²⁴⁰His mutant		
N(δ)	O(H ₂ O)	12.395
N(ε)	O(H ₂ O)	11.666
Glu³⁷⁴His mutant		
N(δ)	O(H ₂ O)	9.631
N(ε)	O(H ₂ O)	9.038
Tyr³⁷⁵Phe mutant		
C(ζ)	O(H ₂ O)	7.724