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

STEFAN MOTIU, VALENTIN GOGONEA
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, \(2H^+ + 2e^- \rightarrow H_2\), 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 archaebacteria 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 acceptors [1–3]. 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 fuel [4], using certain anaerobic organisms [5–8].

This theoretical study aims to find ways of making these enzymes function aerobically (to provide clean fuel, viz., \(H_2\)), because they become inactivated by exogenous ligands such as \(O_2\), \(OH^-\), and \(H_2O\) [9, 10]. Water is the metabolic product of the inactivated catalytic site, that is, \((Fe_d^-)OO \rightarrow (Fe_d^-)H_2O^\cdot\), and it also binds to the hydrogenase active site in its resting state, viz., \(Fe^{II}Fe^{II}\) [11, 12]. By performing Density Functional Theory (DFT) calculations on the \(H\)-cluster, with \(H_2O\), \(OH^-\), and \(O_2\) bound to \(Fe_d\) (redox states \(Fe^{II}-Fe^{III}\)), Liu and Hu [10] 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) [11] and \(Desulfovibrio\) \(desulfuricans\) (DdH), [13] can be used to theoretically investigate their functions via biochemical pathways [10, 14]. Since former DFT and hybrid quantum mechanics/molecular mechanics (QM/MM), calculations [9, 10, 14, 15, 16–22] 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 [9, 10, 14, 15, 19, 20], \(CH_3-S^-\) has been substituted for cysteine, \(Cys^{382}\), and \(H^-\) for the proximal cubane.²

The active site of hydrogenases, viz., the \(H\)-cluster (see Fig. 1), is comprised of a dimetal complex, [Fe-Fe], with the metal centers being bridged by \(di(thiomethyl)amine\) (DTMA), and a cubane subunit, \([Fe_c-S_h]^{2+}\). The iron atoms are coordinated by endogenous ligands, viz., two cyanides (\(CN^-\)), two terminal carbonyls (\(CO_\cdot\)), and a bridging carbonyl (CO),³ An Fe atom, which is part of the proximal
cubane subunit, \([\text{Fe}_4\text{-S}_4]^2+\), is linked to the \(\text{Fe}_p\) of the di-iron subunit, \([\text{Fe}_p\text{-Fe}_d]\), through a cysteinyl sulfur (or \(S_p\) of \(\text{Cys}_{382}\)).

In spite of the di-iron H-cluster subunit redox states, the proximal cubane remains in oxidation state II, \([\text{Fe}_4\text{-S}_4]^2+\), paramagnetic, and catalytically active, where \(\text{Fe}^{1}\text{-Fe}^{1}\), \(\text{EPR}\) silent, is the reduced di-iron H-cluster subunit, \(\text{Fe}^{1}\text{-Fe}^{1}\), paramagnetic, and catalytically active, and \(\text{Fe}^{2}\text{-Fe}^{2}\), \(\text{EPR}\) silent [13, 53], is the fully oxidized, inactive biferrous subunit, and has an \(\text{OH}\) or \(\text{H}_2\text{O}\) molecule bound to the \(\text{Fe}^{2}\text{-Fe}^{2}\).

By performing spectroscopic studies on [Fe-Fe]-hydrogenases, which have been purified from \(\text{Closstridium pasteurianum}\) and \(\text{Desulfovibrio desulfuricans}\), their catalytic functions have been elucidated [11–13, 54, 55]. An X-ray crystal structure of CPI hydrogenase shows an (inactivating) oxygen species that may be \(\text{OH}\) or \(\text{H}_2\text{O}\) bound to the \(\text{Fe}^{2}\) of the H-cluster, while the other X-ray structure has an inactivating CO bound to \(\text{Fe}^{2}\) [11, 12]. For this study, DdH has been selected because its crystal structure has a better resolution (viz., 1.6 Å), than CPI (viz, 1.8 Å) [11, 13].

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, for example, \(\text{CO}\) movement, and the electronic structural methods, such as frontier molecular orbitals (FMO) and natural bond orbital partial charges (NBO).

**Methods**

The ONIOM method [56] (DFT for the QM region, and the universal force field, UFF [57], for the MM region, implemented in Gaussian03 [58]) has been utilized to determine the reaction thermodynamics, viz., \(\Delta G\) 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 [10, 19] in agreement with the 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 6–31G(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 [2,043 H\(_2\)O molecules (in 1 nm layer peripheral to H-cluster]), six Na\(^+\) ions were randomly incorporated into the solvent (7 Na\(^+\) ions for clusters 1, 3, 4; 8 Na\(^+\) ions for cluster 2) to neutralize the negative charges [23] on the H-cluster, medial, and distal cubane/cysteines clusters.\(^4\) 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 two 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 three 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 \(\text{Fe}_p\) and...
CO<sub>p-p</sub> in the QM region have been kept frozen. The rational for freezing Fe<sub>p</sub> and CO<sub>p-p</sub> arises from former optimizations [15] 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., ΔG. The reactivation pathways (Schemes 1–3) proceed with different combinations for the three steps. For the hybrid calculations, the reductive step, 1 → 2 (Scheme 1), of [Fe<sup>III</sup>-Fe<sup>III</sup>]-hydrogenase H-cluster proceeds rather endergonically, viz., ΔG<sub>Enzyme</sub> = +42.6 kcal/mol (Table I) relative to the gas phase (ΔG<sub>Gas</sub> = +8.2 kcal/mol, Table I), 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 → 3 (Scheme 1), is highly exergonic (ΔG<sub>Enzyme</sub> = −317.9 kcal/mol, see Supporting Information Table I 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 I) is +91.3 kcal/mol, and points to a more spontaneous reaction in gas phase. In step 3 → 4 (H<sub>2</sub>O elimination), the hydrogenase H-cluster calculations show a rather small, endergonic result (ΔG<sub>Enzyme</sub> Φ = +2.0 kcal/mol), as opposed to the exergonic gas phase outcome (ΔG<sub>Gas</sub> Φ = −6.6 kcal/mol). The enzymatic H<sub>2</sub>O removal is non-spontaneous due to the influence of the protein environment on the H-cluster electronic properties. The DdH protonation, 1 → 2′ (Scheme 2), provides an exergonic reaction, viz., ΔG<sub>Enzyme</sub> Φ = −236.8 kcal/mol, proceeding less spontaneously than in gas phase, viz. ΔG<sub>Gas</sub> Φ = −328.3 kcal/mol, correlating the trend in Scheme 1. The enzyme Φ calculations, for the H<sub>2</sub>O removal, 2′ → 3′; (Scheme 2), confer an endergonic result, (ΔG<sub>Enzyme</sub> Φ = +22.9 kcal/mol), as opposed to the exergonic gas Φ.
reaction step ($\Delta G_{\text{Gas}} = -16.6 \text{ kcal/mol}$), which results in a difference of $+39.5 \text{ kcal/mol}$. 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 (Scheme 2) proceeds exergonically, viz., $\Delta G_{\text{Enzyme}} = -59.4 \text{ kcal/mol}$, close to the gas phase result, viz., $\Delta G_{\text{Gas}} = -62.7 \text{ kcal/mol}$.

However, Scheme 3, in contrast, definitely provides room for enhancements that could achieve H$_2$O removal because, in step $3 \rightarrow 4$, the hydrogenase H-cluster calculations provide a rather small, free energy, viz., $\Delta G_{\text{Enzyme}} = +2.0 \text{ kcal/mol}$, which could be changed into an exergonic reaction via mutagenesis. For Scheme 3, only the H$_2$O removal step is endergonic (viz., $\Delta G_{\text{Enzyme}} = +2.0 \text{ kcal/mol}$, as in Scheme 1), whereas the other remaining steps [protonation (viz., $\Delta G_{\text{Enzyme}} = -236.8 \text{ kcal/mol}$), reduction (viz., $\Delta G_{\text{Enzyme}} = -38.4 \text{ kcal/mol}$)] are exergonic.

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 H$_2$O.

The wild-type hydrogenase has been residue manipulated in two ways. First of all, the considered residues (Table I) 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., Arg$^{111}$Glu, Thr$^{145}$Val, Ser$^{177}$Ala, Glu$^{240}$His, Glu$^{374}$His, and Tyr$^{375}$Phe.

In Scheme 1, the reduction step, $1 \rightarrow 2$, upon the removal of Glu$^{240}$, becomes less endergonic by $\Delta G = +17.0 \text{ kcal/mol}$ (relative to wild-type DdH) but not even close to make it spontaneous.$^7$ For $2 \rightarrow 3$, with the removal of a basic residue, that is, Lys$^{237}$ (when compared with the wild-type enzyme), the protonation step proceeds more exergonically, viz., by a Gibbs’ energy difference of $+27.3 \text{ kcal/mol}$. The last step, $3 \rightarrow 4$, proceeds exergonically, viz., $\Delta G_{\text{Enzyme}} = -1.6 \text{ kcal/mol}$, upon the removal of Glu$^{374}$ from the apoenzyme; however, Scheme 1
cannot proceed to the completion due to the endergonic, reductive step, $1 \rightarrow 2$.

For Scheme 2, an improvement has been obtained (as in Scheme 1) upon the removal of exactly the same residue for each of the corresponding step, that is, Lys$^{237}$ for protonation (viz., $\Delta G_{\text{Enzyme}} \Phi = -264.4$ kcal/mol), Glu$^{240}$ for reduction (viz., $\Delta G_{\text{Enzyme}} \Phi = -78.7$ kcal/mol), and Glu$^{374}$ for H$_2$O elimination (viz., $\Delta G_{\text{Enzyme}} \Phi = +21.5$ kcal/mol). However, in spite of Gibbs' energy improvements for all reaction steps, no matter what residues (Table I) are removed, Scheme 2 is hindered from completion in the H$_2$O removal step.

In Scheme 3, the only endergonic step is for the removal of H$_2$O ($\Delta G = +2.0$ kcal/mol), which was, nevertheless, made to proceed exergonically by eliminating several residues, one at a time. The most promising residues are Arg$^{111}$, Thr$^{145}$, Ser$^{177}$, Glu$^{240}$, Glu$^{374}$, and Tyr$^{375}$, 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 I). In $3 \rightarrow 4$, single residue substitutions have been carried out on the six residues that hinder the removal of water, viz., Arg$^{111}$Glu, Thr$^{145}$Val, Ser$^{177}$Ala, Glu$^{240}$His, Glu$^{374}$His, and Tyr$^{375}$Phe, followed by (sequential) frequency calculations.

Then out of the most successful substitutions, two, three, and four residue combinations were examined by frequency calculations (Table II). The 1st two-residue combination is Glu$^{374}$His and Tyr$^{375}$Phe giving $\Delta G = -5.1$ kcal/mol; the 2nd three-residue combination is Thr$^{145}$Val, Glu$^{374}$His, and Tyr$^{375}$Phe giving $\Delta G = -6.2$ kcal/mol, and the 3rd four-residue combination is Arg$^{111}$Glu, Thr$^{145}$Val, Glu$^{374}$His, and Tyr$^{375}$Phe giving $\Delta G = -7.5$ kcal/mol.

GEOMETRIC CONSIDERATIONS

To explain Gibbs’ energies between the aqueous enzyme $\Phi$ and the gas $\Phi$ calculations, DdH H-cluster and H-cluster geometries for the two phases are analyzed, and then the hydrogenase H-cluster
distances to the six replaced, juxtaposed residues are presented for the aqueous enzyme $\Phi$.

The wild-type DdH QM/MM calculations for $\text{H}_2\text{O}$ removal, $(3 \rightarrow 4)$, reveal a contrasting picture regarding CO$_b$ translation towards Fe$_d$ relative to the gas phase $\text{H}_2\text{O}$ elimination. That is, in Table III, the iron-carbon distance, Fe$_d$-CO$_b$, essentially remains constant [(viz., 1.907 Å $\rightarrow$ 1.908 Å), whereas the gas $\Phi$ distance becomes smaller (viz., 1.945 Å $\rightarrow$ 1.850 Å), and the reaction $(3 \rightarrow 4)$ is exergonic], [14, 15] which may explain why $\text{H}_2\text{O}$ removal is endergonic for the enzyme $\Phi$.

Thus, it is ascertained that for the aqueous enzyme $\Phi$, an endergonic ligand ($\text{H}_2\text{O}$) dissociation is manifested in the Fe$_d$-CO$_b$ bond contraction (1.959 Å $\rightarrow$ 1.939 Å). The opposite trend is observed in gas phase, where the Fe$_d$-CO$_b$ bond is elongated (2.013 Å $\rightarrow$ 2.232 Å) after $\text{H}_2\text{O}$ elimination.

Out of the three reaction pathways presented here, only Scheme 3 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 $\text{H}_2\text{O}$, before and after water removal, to compare the $\text{H}_2\text{O}$ elimination Gibbs’ energies for the residue substituted DdH with the thermodynamics of the wild-type DdH.

For the removal of water from the wild-type enzyme, the distance between Fe$_d$ and CO$_b$ is slightly increasing (Table III), which corresponds to the endergonic step, viz., $\Delta G_{\text{Enzyme}} = +2.0$ kcal/mol. However, the $\text{H}_2\text{O}$ removal from the mutated enzyme is exergonic (Table II), 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 Arg$^{111}$Glu, 0.028 Å for...
and III. have been obtained (ca. 0.1 Å, Table III). It is also bond contractions between the iron atoms, Fe6-Fep, corresponding to exergonic steps for water elimination.

Reaction steps & (+e\(^{-}\)) & (+H\(^{+}\)) & (-H\(_{2}\)O) & (+H\(^{+}\)) & (-H\(_{2}\)O) & (+e\(^{-}\)) & (+e\(^{-}\)) \\
Native DdH & 1 \to 2 & 2 \to 3 & 3 \to 4 & 1 \to 2' & 2' \to 3' & 3' \to 4 & 2' \to 3 \\
\(\Delta\)Ser\(^{62}\) & +42.6 & -317.9 & +2.0 & -236.8 & +22.9 & -59.4 & -38.4 \\
\(\Delta\)Arg\(^{111}\) & +38.3 & -313.6 & +2.0 & -232.6 & +23.1 & -63.9 & -42.7 \\
\(\Delta\)Tyr\(^{112}\) & +59.0 & -323.4 & -0.7 & -251.4 & +22.0 & -44.7 & -22.0 \\
\(\Delta\)Asp\(^{144}\) & +47.5 & -322.1 & +1.6 & -241.1 & +22.8 & -54.8 & -33.6 \\
\(\Delta\)Thr\(^{145}\) & +28.8 & -305.0 & +3.0 & -223.4 & +24.1 & -73.8 & -52.8 \\
\(\Delta\)Glu\(^{146}\) & +44.1 & -318.8 & -0.2 & -237.9 & +22.8 & -59.9 & -36.9 \\
\(\Delta\)Thr\(^{148}\) & +32.5 & -308.5 & +2.5 & -227.4 & +23.6 & -69.7 & -48.6 \\
\(\Delta\)Ser\(^{150}\) & +41.8 & -316.8 & +1.8 & -235.8 & +22.9 & -60.3 & -39.2 \\
\(\Delta\)Asp\(^{152}\) & +32.8 & -308.1 & +2.1 & -227.0 & +23.3 & -69.5 & -48.3 \\
\(\Delta\)Thr\(^{155}\) & +47.1 & -322.6 & +1.9 & -241.6 & +23.0 & -55.1 & -33.9 \\
\(\Delta\)Glu\(^{158}\) & +36.5 & -311.4 & +1.9 & -230.2 & +23.0 & -65.8 & -44.7 \\
\(\Delta\)Thr\(^{176}\) & +43.8 & -319.5 & +2.1 & -238.3 & +23.2 & -58.5 & -37.4 \\
\(\Delta\)Ser\(^{177}\) & +38.3 & -312.8 & -0.5 & -231.8 & +22.9 & -66.1 & -42.7 \\
\(\Delta\)Gln\(^{183}\) & +46.3 & -322.3 & +2.3 & -241.1 & +23.3 & -55.9 & -34.9 \\
\(\Delta\)Ser\(^{198}\) & +42.6 & -318.0 & +2.1 & -236.9 & +23.2 & -59.6 & -38.5 \\
\(\Delta\)lys\(^{201}\) & +48.3 & -323.5 & +1.7 & -242.6 & +22.7 & -53.6 & -32.6 \\
\(\Delta\)Ser\(^{202}\) & +46.3 & -324.1 & +2.4 & -241.8 & +22.5 & -56.0 & -35.9 \\
\(\Delta\)Asn\(^{207}\) & +46.7 & -321.6 & +1.5 & -240.6 & +22.5 & -55.2 & -34.2 \\
\(\Delta\)Ser\(^{230}\) & +38.2 & -314.0 & +2.3 & -232.9 & +23.3 & -64.0 & -42.9 \\
\(\Delta\)lys\(^{237}\) & +69.2 & -345.2 & +1.2 & -264.4 & +21.7 & -32.1 & -11.6 \\
\(\Delta\)lys\(^{238}\) & +47.4 & -322.1 & +1.7 & -241.5 & +22.9 & -54.4 & -33.3 \\
\(\Delta\)Glu\(^{240}\) & +25.6 & -300.1 & +0.8 & -218.9 & +23.8 & -78.7 & -55.6 \\
\(\Delta\)Thr\(^{257}\) & +42.8 & -318.0 & +1.9 & -236.9 & +23.1 & -59.5 & -38.3 \\
\(\Delta\)Thr\(^{259}\) & +45.9 & -320.8 & +1.8 & -239.9 & +23.0 & -56.1 & -35.0 \\
\(\Delta\)Thr\(^{260}\) & +42.1 & -317.4 & +2.0 & -236.4 & +23.1 & -60.1 & -39.0 \\
\(\Delta\)Ser\(^{289}\) & +44.0 & -319.1 & +1.9 & -238.1 & +22.9 & -58.1 & -37.0 \\
\(\Delta\)Thr\(^{294}\) & +40.4 & -316.0 & +2.2 & -234.9 & +23.2 & -61.8 & -40.8 \\
\(\Delta\)Thr\(^{299}\) & +40.9 & -316.2 & +1.9 & -235.1 & +23.0 & -61.2 & -40.1 \\
\(\Delta\)Glu\(^{374}\) & +27.7 & -301.3 & -1.6 & -220.2 & +21.5 & -76.5 & -53.4 \\
\(\Delta\)Tyr\(^{375}\) & +41.5 & -315.8 & +1.1 & -234.8 & +22.3 & -60.6 & -39.5 \\
\(\Delta\)Gln\(^{388}\) & +44.0 & -319.2 & +2.0 & -238.1 & +23.1 & -58.2 & -37.1 \\
Gas \(\phi\) & +8.2 & -409.2 & -6.6 & -328.3 & -16.6 & -62.7 & -72.7 \\

\(^a\) Residue removed DdH. \\
\(s\) = small chain.

Note that a simultaneous bond elongation occurs [for all presented mutations (Table III)] between the Fe\(_p\) and the bridging carbonyl, Fe\(_p\)-CO\(_p\), when bond contraction for Fe\(_p\)-CO\(_p\) takes place. As a result of mutating DdH (vs. the wild-type enzyme), larger bond contractions between the iron atoms, Fe\(_p\)-Fe\(_p\), have been obtained (ca. 0.1 Å, Table III). It is also noticed that the bond length between the distal iron and water, Fe\(_p\)-H\(_2\)O, is longer, that is, 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 (Table IV), the more spontaneous is the water removal step becomes. The exception is Tyr\(^{375}\)Phe, in which the substituted amino acid is highly hydrophobic; although it gets closest to the exogenous oxygen atom of H\(_2\)O, it nevertheless has less effect on removing water when compared with Glu\(^{374}\)His mutant where the substituted amino acid (at a greater
distance from H₂O is not only polar but also of opposite charge as well. The negative partial charge on oxygen (q_{\text{COO}} = -0.935) of H₂O repels the negatively charged carboxylate of Glu^{374} (q_{\text{COO}} = 0.547, -0.567, -0.563), thus making the water removal difficult. When Glu^{374} 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 proxim-

### TABLE II
DdH Gibbs' energies (kcal/mol) of one, two, three, and four residue mutations for reaction step (3 → 4).

<table>
<thead>
<tr>
<th>Residue substitutions for H₂O removal step 3 → 4</th>
<th>ΔH (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg^{111}Glu</td>
<td>-0.9</td>
</tr>
<tr>
<td>Thr^{149}Val</td>
<td>-1.3</td>
</tr>
<tr>
<td>Ser^{377}Ala</td>
<td>0.1</td>
</tr>
<tr>
<td>Glu^{240}His</td>
<td>1.1</td>
</tr>
<tr>
<td>Glu^{374}His</td>
<td>-3.1</td>
</tr>
<tr>
<td>Tyr^{375}Phe</td>
<td>-2.1</td>
</tr>
<tr>
<td>Combinations of Glu^{374}His and Tyr^{375}Phe</td>
<td>-5.1</td>
</tr>
<tr>
<td>Thr^{149}Val, Glu^{374}His, and Tyr^{375}Phe</td>
<td>-6.2</td>
</tr>
<tr>
<td>Arg^{111}Glu, Thr^{149}Val, Glu^{374}His, and Tyr^{375}Phe</td>
<td>-7.5</td>
</tr>
</tbody>
</table>

### TABLE III
Interatomic distances (Å) for wild-type and mutated DdH, between Feₚ, Feₜ, Feₚ, and CO𝑏, Feₜ and CO𝑏, and Feₚ and H₂O, before and after H₂O removal (3 → 4).

<table>
<thead>
<tr>
<th>Before H₂O removal</th>
<th>3 (Wild-type)</th>
<th>3 (Arg^{111} Glu mutant)</th>
<th>3 (Thr^{149} Val mutant)</th>
<th>3 (Glu^{374} His mutant)</th>
<th>3 (Tyr^{375} Phe mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feₚ-Feₜ</td>
<td>2.626</td>
<td>2.659</td>
<td>2.670</td>
<td>2.690</td>
<td>2.666</td>
</tr>
<tr>
<td>COₚ-Feₚ</td>
<td>1.959</td>
<td>1.963</td>
<td>1.968</td>
<td>1.975</td>
<td>1.991</td>
</tr>
<tr>
<td>COₜ-Feₚ</td>
<td>1.907</td>
<td>1.952</td>
<td>1.963</td>
<td>1.945</td>
<td>1.937</td>
</tr>
<tr>
<td>Feₚ-O(H₂O)</td>
<td>2.127</td>
<td>2.205</td>
<td>2.181</td>
<td>2.223</td>
<td>2.184</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>After H₂O removal</th>
<th>4 (Wild-type)</th>
<th>4 (Arg^{111} Glu mutant)</th>
<th>4 (Thr^{149} Val mutant)</th>
<th>4 (Glu^{374} His mutant)</th>
<th>4 (Tyr^{375} Phe mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feₚ-Feₜ</td>
<td>2.587</td>
<td>2.584</td>
<td>2.616</td>
<td>2.589</td>
<td>2.582</td>
</tr>
<tr>
<td>COₚ-Feₚ</td>
<td>1.939</td>
<td>2.006</td>
<td>2.003</td>
<td>2.008</td>
<td>2.005</td>
</tr>
<tr>
<td>COₜ-Feₚ</td>
<td>1.908</td>
<td>1.928</td>
<td>1.935</td>
<td>1.928</td>
<td>1.906</td>
</tr>
</tbody>
</table>

### TABLE IV
Interatomic distances between the oxygen (of exogenous H₂O; Feₚ-OH₂, compound 3) and the juxtaposed atoms of the residue R-groups.

<table>
<thead>
<tr>
<th>Mutated DdH</th>
<th>H₂O oxygen</th>
<th>Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg^{111}Glu mutant</td>
<td>O(\text{H})</td>
<td>17.507</td>
</tr>
<tr>
<td>O(\text{H})</td>
<td>O(H₂O)</td>
<td>18.259</td>
</tr>
<tr>
<td>Thr^{149}Val mutant</td>
<td>C(\gamma)</td>
<td>9.616</td>
</tr>
<tr>
<td>C(\gamma)</td>
<td>O(H₂O)</td>
<td>9.650</td>
</tr>
<tr>
<td>Ser^{377}Ala mutant</td>
<td>C(\beta)</td>
<td>6.077</td>
</tr>
<tr>
<td>C(\beta)</td>
<td>O(H₂O)</td>
<td>12.395</td>
</tr>
<tr>
<td>Glu^{240}His mutant</td>
<td>N(\delta)</td>
<td>11.666</td>
</tr>
<tr>
<td>N(\delta)</td>
<td>O(H₂O)</td>
<td>9.631</td>
</tr>
<tr>
<td>Glu^{374}His mutant</td>
<td>N(\epsilon)</td>
<td>9.038</td>
</tr>
<tr>
<td>N(\epsilon)</td>
<td>O(H₂O)</td>
<td>7.724</td>
</tr>
</tbody>
</table>

| Tyr^{375}Phe mutant          | C(\iota)   |   |
| C(\iota)                    | O(H₂O)     |   |
ity to a closed-shell H-cluster, the resulting $\sigma$-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 1, 1 $\rightarrow$ 2), and according to the LUMO depiction (see Fig. 2), the transferred $e^-$ appears to be localized in the vicinity of the di-iron atoms, Fe_p-Fe_q. However, according to the NBO partial charge results (see Fig. 3), the iron atoms (of hydrogenase H-cluster 1) do not have affinity for the approaching $e^-$, that is, $q_{Fe_p} = -1.291$ a.u. and $q_{Fe_q} = -1.109$ a.u. The fact that the reduction is endergonic ($\Delta G_{\text{Enzyme}} = +42.6$ 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_p, HOMO_p, and HOMO_o, have similar orbital distribution over OH (Fig. 2), and could make a $\sigma$-bond with the incoming H$^+$; in conjunction with the NBO partial charge of the hydroxyl oxygen, $q_O = -0.991$ a.u., the large H$^+$ affinity for cluster 2, $\Delta G = -317.9$ kcal/mol, (2 $\rightarrow$ 3) comes as no surprise.

In H-cluster 3, (3 $\rightarrow$ 4), the open shells, HOMO_p, HOMO_o, and SOMO_p, are diffused throughout the cluster except over the Fe_p-OH_2 bond, implying that the $e^-$ of the $\sigma$-bond, Fe_p-OH_2, reside in a lower energy state, which also explains the negative
NBO partial charges of both Fe_d ($q_{Fe_d} = -1.012$ a.u.), and the O ($q_O = -0.935$ a.u.) of H$_2$O. This fact may explain, therefore, the affinity of Fe$_d$ for H$_2$O, that is, $\Delta G = +2.0$ kcal/mol. For [Fe-Fe]-hydrogenase H-cluster 1, (Scheme 2, 1 $\rightarrow$ 2'), the HOMO is more diffused over Fe$_{d'}$, DTMA bidentate ligand, and over the exogenous ligand, that is, 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 ($q_O = -0.898$; $q_N = -0.682$). Note that these analyses also agree with the calculated hydrogenase H-cluster proton affinity, that is, $\Delta G = -236.8$ kcal/mol.

For H-cluster 2', (Scheme 2, 2' $\rightarrow$ 3'), HOMO is diffused over the center of Fe$_p$-Fe$_d$ subcluster, CO$_{d'}$, and over CN$^-$ (Fe$_o$ coordinated) which means that the $e^-$ of the $\sigma$-bond, Fe$_{d'}$-OH$_2$, are situated in a lower energy state, explaining the negative NBO partial charges of both O (of H$_2$O, $q_O = -0.878$ a.u.) and Fe$_d$ ($q_{Fe_d} = -1.077$ a.u.). This, then, accounts for the bond strength of Fe$_d$ for H$_2$O, that is, $\Delta G = +22.9$ 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 1, 3 $\rightarrow$ 4)], which partially accounts for the more endergonic H$_2$O 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, that is, $q_{Fe_p} = -1.317$ 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, that is, $\Delta G_{Enzyme} \Phi = -59.4$ kcal/mol, although the di-iron atoms have rather high NBO charges.

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$_b$ (Fig. 2). Cluster reduction (Scheme 3, 2' $\rightarrow$ 3) occurs with a rather high spontaneity, that is, $\Delta G_{Enzyme} \Phi = -38.4$ kcal/mol, although the di-iron atoms have high NBO negative charges, that is, $q_{Fe_p} = -1.305$ 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 1, 1 $\rightarrow$ 2, perhaps because the total charge on each cluster is different, that is, cluster 2' has charge 0 a.u., whereas 1 has a charge of $-1$ a.u.  

Discussion

The comparison between gas phase and wild-type enzyme calculations, presented in this 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 COb away from the catalytic site, Fe$_d$, leading to an exogenously inhibited hydrogenase, and thus hindering H$_2$O elimination. On the contrary, suitable residue substitutions can reverse the enzyme inhibition by al-

Because 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$_{S4}$/Cys$_d$), cluster.
allowing CO₂ to migrate towards Fe₄₅, concomitant with H₂O removal.

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

Also, the reactivation pathway of [Fe-Fe]-hydrogenase H-cluster 4 (Scheme 2) cannot be realized because of the high nonsensitiveness step for H₂O removal (2′ → 3′), ΔG_{Enzyme} = +22.9 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 3 provides the best chance for reactivating DdH because the only reaction step to overcome (H₂O removal) is barely endergonic (ΔG_{Enzyme} = +2.0 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 ΔG = −7.5 kcal/mol).

Conclusions

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

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

Pathway III, however, proceeds by an exergonic protonation step (viz., ΔG_{Enzyme} = −236.8 kcal/mol), an exergonic e⁻ transfer step (viz., ΔG_{Enzyme} = −38.4 kcal/mol), followed by water removal step (viz., ΔG_{Enzyme} = +2.0 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 ΔArg¹¹¹, ΔThr¹⁴⁵, ΔSer¹⁷⁷, ΔGlu²⁴⁰, ΔGlu³⁷⁴, and ΔTyr³⁷⁵, and their respective Gibb’s 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 ΔSer¹⁷⁷ → Ser¹⁷⁷Ala (ΔG = −0.5 kcal/mol → ΔG = −0.1 kcal/mol), and ΔGlu²⁴⁰ → Glu²⁴⁰His (ΔG = +0.8 kcal/mol → ΔG = +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, Glu²⁴⁰His and Tyr³⁷⁵Phe, resulted in AG = −5.1 kcal/mol; the three residue combination, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe, provided a ΔG = −6.2 kcal/mol, and the four residue combination, Arg¹¹¹Glu, Thr¹⁴⁵Val, Glu³⁷⁴His, and Tyr³⁷⁵Phe, gave a ΔG = −7.5 kcal/mol. Although the H₂O removal thermodynamic trend is not precisely cumulative, it seems to point in that direction.

The wild-type DdH H-cluster bond distance, that is, Fe₄⁺CO₂, 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 Φ² 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.

Because 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
$\Delta G_{\text{enzyme}} = +2.0 \text{ kcal/mol}$ shows that the distance between the Fe$_4$ and CO$_b$ remains approximately the same. However, the H$_2$O removal from the mutated enzyme proceeds exergonically, correlating to the movement of the CO$_b$ towards the Fe$_4$. The following bond contractions, Fe$_4$-CO$_b$, have been obtained, viz., 0.024 Å for Arg$_{111}$Glu, 0.028 Å for Thr$_{145}$Val, 0.017 Å for Glu$_{374}$His, and 0.031 Å for Tyr$_{375}$Phe, corresponding to exergonic steps for water elimination. Additionally, bond contractions have been obtained between the iron atoms (ca. 0.1 Å), concurrent with H$_2$O dissociation.

We conclude by postulating that even a single and proper residue (experimental) substitution, like Glu$_{374}$Glu$_{374}$His ($\Delta G = -1.6 \text{ kcal/mol}$, $\Delta G = -3.1 \text{ kcal/mol}$), 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.

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References

64. Guex, N.; Peitsch, M. C. Electrophoresis 1997, 18, 2714.