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A Theoretical Study for the Reactivation of O2 Inhibited [Fe-Fe]-Hydrogenase

Stefan Motiu
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A THEORETICAL STUDY FOR THE REACTIVATION OF $O_2$

INHIBITED [FE-FE]-HYDROGENASE

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Bachelor of Science in Mathematics
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August, 2002

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A THEORETICAL STUDY FOR THE REACTIVATION OF O\textsubscript{2} INHIBITED [FE-FE]-HYDROGENASE

STEFAN MOTIU

ABSTRACT

The current investigation presents a reactivation pathway of the exogenously inhibited H-cluster (viz., by O\textsubscript{2}, or OH\textsuperscript{-}, which metabolizes to H\textsubscript{2}O), for both vacuum and aqueous enzyme phase. The H-cluster is the catalytic site of [Fe-Fe]-hydrogenase, with the latter extracted from Desulfovibrio desulfuricans (Dd) bacteria.

It consists of proximal iron, Fe\textsubscript{p}, and distal Fe\textsubscript{d} subunit, [Fe\textsubscript{p}-Fe\textsubscript{d}], which is bridged by di(thiomethyl)amine (DTMA) ligand, and a proximal cubane subunit, [Fe\textsubscript{4}-S\textsubscript{4}]\textsuperscript{2+}. [Fe\textsubscript{p}-Fe\textsubscript{d}] is coordinated by two cyanides (CN\textsuperscript{-}), two terminal carbonyls (CO\textsubscript{t}), and a bridging carbonyl (CO\textsubscript{b})\textsuperscript{α}. An Fe atom from [Fe\textsubscript{4}-S\textsubscript{4}]\textsuperscript{2+} connects Fe\textsubscript{p} through a cysteinyl sulfur (of Cys\textsubscript{382}).

Density functional theory calculations on the native and ruthenium-modified H-cluster (gas phase) have been performed using the B3LYP functional with 6-31+G** and 6-311+G** bases sets. We have ascertained that there is a thermodynamically favorable pathway for the reactivation of the OH\textsuperscript{-} inhibited H-cluster, which proceeds by an initial protonation of Fe\textsubscript{d}-OH\textsuperscript{-} complex. The proposed reaction pathway has all of its intermediate reactions proceed exergonically.

The aqueous enzyme phase investigation uses the hybrid quantum mechanics/molecular mechanics (QM/MM) method to study reactivation pathways for

\textsuperscript{α}The di-iron atoms are named proximal and distal, Fe\textsubscript{p}-Fe\textsubscript{d} (for Fe\textsubscript{p} is closest to the ‘proximal’ cubane, while Fe\textsubscript{d} is ‘distal’ from the cubane).
the exogenously inhibited enzyme matrix. ONIOM calculations performed on the enzyme agree with experimental results, i.e., the hydrogenase H-cluster is inhibited by oxygen metabolites.

To investigate potential inhibitory residues that prevent H$_2$O from leaving the catalytic site, and reactivate the hydrogenase H-cluster, an enzyme spherical region of radius 8 Å (from the distal iron, Fe$_d$, of [Fe-Fe]-hydrogenase H-cluster) was screened. In the screening process, polar residues were removed, one at a time, and frequency calculations provided the change in Gibbs’ energy of water dissociation (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 of H$_2$O elimination. Favorable thermodynamic results have been obtained for both single residue removal ($\Delta G_{\text{Glu}^{374}} = -1.6$ kcal/mol), single substitution ($\Delta G_{\text{Glu}^{374}\text{His}} = -3.1$ kcal/mol), and combined residue substitutions ($\Delta G_{\text{Arg}^{111}\text{Glu}; \text{Thr}^{145}\text{Val}; \text{Glu}^{374}\text{His}; \text{Tyr}^{375}\text{Phe}} = -7.5$ kcal/mol). Because the wild-type enzyme has only an endergonic step to overcome, i.e., for H$_2$O removal, by eliminating several residues, one by one, the endergonic step was made to proceed more spontaneously. Thus, the most promising residue deletions which enhance H$_2$O elimination are $\Delta \text{Arg}^{111}$, $\Delta \text{Thr}^{145}$, $\Delta \text{Ser}^{177}$, $\Delta \text{Glu}^{240}$, $\Delta \text{Glu}^{374}$, and $\Delta \text{Tyr}^{375}$.

Hence, both single and combined residue substituted [Fe-Fe]-hydrogenase show increased spontaneity for H$_2$O removal. The thermodynamics and electronic structure analyses show that CO$_b$ 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 CO₆ towards Fe₄, which leads to enzyme inhibition. But enzyme reactivation can be achieved by suitable residue substitutions.
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CHAPTER I

[FE-FE]-HYDROGENASE BACKGROUND

1.1. General Considerations

There are two fundamental objectives that the current research endeavors to achieve. First, it seeks to find a reaction pathway, in gas phase, for the reactivation of exogenously inhibited H-cluster, which is the catalytic site of [Fe-Fe]-hydrogenase. Then, it aims to ascertain a reaction pathway, in aqueous enzyme phase, for the reactivation of the inactivated [Fe-Fe]-hydrogenase from Desulfovibrio desulfuricans (Dd). The H-cluster is the catalytic site of Desulfovibrio desulfuricans hydrogenase (DdH) which is aerobically inactivated.

It is hypothesized that alternative reaction mechanism(s) must exist by which the hydrogenase can be reactivated. Hence, in order to determine the reactivation
mechanism(s), quantum mechanics (QM) calculations (in vacuum), and hybrid quantum mechanics and molecular mechanics methods (QM/MM) (in aqueous phase) were carried out.

(i) An objective of the current research is to find at least a reaction pathway, in gas phase, for the reactivation of the exogenously inhibited H-cluster (the active site of [Fe-Fe]-hydrogenases, Chapter II).

The rationale is that if there exists at least a thermodynamically favorable pathway (on the free energy surface of the H-cluster) for the reactivation of the inhibited H-cluster by OH\(^-\), or H\(_2\)O, then it ought to be found computationally. Moreover, the potential reaction pathway ought to have its intermediate reactions ensue exergonically.

Density functional theory (DFT) calculations were performed on the [Fe-Fe] and Ru-modified H-cluster to determine whether specific bimetal combinations can improve the reactivation mechanism. Thermodynamic and geometric analysis were carried out regarding the inhibition of both [Fe-Fe] and Ru-modified H-cluster.

(ii) Another desideratum is to find no less than a reaction pathway, in aqueous enzyme phase, for the reactivation of the exogenously inhibited [Fe-Fe]-hydrogenases (Chapter III, IV).

Because gas phase exergonic reaction pathway results have been successfully obtained, the justification in this case is that inactivation-removal mechanism studies of [Fe-Fe]-hydrogenase H-cluster using ONIOM should also be successful in determining (residue mutated) oxidation-resistant DdH.

DdH residue eliminations and substitutions have been carried out which corroborated that the mutated DdH can be reactivated.
1.2. Introduction

Hydrogenases were first discovered in the 1930s. Subsequently, theoretical and experimental scientists became interested in synthesizing hydrogenase H-cluster analogues in order to understand their catalytic mechanism\textsuperscript{1,2}, which in turn could help them produce clean, potential energy-H\textsubscript{2}\textsuperscript{3}.

[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\textsuperscript{+} + 2e\textsuperscript{−} ⇌ H\textsubscript{2}, which occurs in anaerobic media. In living systems, of the two metalloproteins, [Fe-Fe]-hydrogenases are mostly used for H\textsubscript{2} production\textsuperscript{4} having a reactivity of about 2 orders of magnitude larger than [Ni-Fe]-hydrogenases. [Fe-Fe]-hydrogenases are either monomeric and located cytoplasmically, or heterodimeric and found periplasmatically\textsuperscript{5}. They are found in many bacteria, simple eukaryotes, and archaea where they provide H\textsubscript{2} for the metabolic processes of these life forms. By means of H\textsubscript{2} oxidation, ATP synthesis exploits H\textsubscript{2} as an energy source, whereas H\textsubscript{2} synthesis results from the metabolic disposal of excess electron (with available protons), or from pyruvate fermentation. Proteins, such as ferredoxins, cytochrome C\textsubscript{3}, and cytochrome C\textsubscript{6}, act as physiological e\textsuperscript{−} donors or acceptors (Figure 1-1, 1-2, 1-3)\textsuperscript{6-8}.

The exploration of alternative energy sources has kindled great interest in hydrogenase research. The reason for studying biological H\textsubscript{2} production is to clarify the complex mechanism (for hydrogen synthesis), which may help researchers produce clean fuel\textsuperscript{9}, using certain anaerobic organisms\textsuperscript{10-11}. 
Figure 1-1. Model of electron transfer between DdH and c553


An Electron Transfer Pathway in [Fe-Fe]-hydrogenase

Figure 1-2. Best pathway coupling value: $2.092 \times 10^{-7}$ (calculated by HARLEM software program$^{58}$).
A Different Electron Transfer Pathway in [Fe-Fe]-hydrogenase

Figure 1-3. Another pathway coupling value: $2.580 \times 10^{-7}$ (calculated with HARLEM program\textsuperscript{58}).
This theoretical study aims to find ways of making these enzymes function aerobically\textsuperscript{2,3} (to provide clean fuel, viz., $H_2$\textsuperscript{a}), because they become inactivated by exogenous ligands such as $O_2$, $OH^-$, and $H_2O$\textsuperscript{12,13}.

Water is the metabolic product of the inactivated catalytic site, i.e., $(Fe_{d-})OO \rightarrow (Fe_{d-})H_2O$, and it also binds to the hydrogenase active site in its resting state, viz., $Fe^{II}Fe^{III}$\textsuperscript{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 $Fe^{II}$-$Fe^{III}$), Liu and Hu\textsuperscript{3} 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 \textit{Clostridium pasteurianum} (CPI)\textsuperscript{12} and \textit{Desulfovibrio desulfuricans} (Dd)\textsuperscript{14}, can be used to theoretically investigate their functions via biochemical pathways\textsuperscript{3,15}. Since former DFT, and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations\textsuperscript{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 investigation, as well as in other computational studies\textsuperscript{1-3,15,19,20}, $CH_3$-$S^-$ has been substituted for cysteine, Cys\textsuperscript{382}, and $H^+$ for the proximal cubane\textsuperscript{8}.

\textsuperscript{a}In fuel cells, the product, $(H_2 + \frac{1}{2}O_2 \rightarrow) H_2O$, is benign relative to the current, carcinogenic hydrocarbon combustion emissions.

\textsuperscript{b}A $H^+$ is replaced for $[Fe_{d-}S_4]^{2+}$, and $CH_3$-$S^-$ for cysteine-$S^-$ in order to minimize computational time, and cost.
1.3. [Fe-Fe]-hydrogenase active site: the H-cluster

The active site of hydrogenases, viz., the H-cluster (Figure 1-4), is comprised of a dimetal complex, [Fe-Fe], with the metal centers being bridged by di(thiomethyl)amine (DTMA), and a cubane subunit, [Fe₄-S₄]²⁺. The iron atoms are coordinated by endogenous ligands, viz., two cyanides (CN⁻), two terminal carbonyls (CO₉), and a bridging carbonyl (CO₈). An Fe atom, which is part of the proximal cubane subunit, [Fe₄-S₄]²⁺, is linked to the Feₚ of the di-iron subunit, [Feₚ-Feₐ], through a cysteiny!l sulfur (or Sγ of Cys³⁸²).

1.4. [Fe-Fe]-hydrogenase H-cluster coordination and redox states

In spite of the di-iron H-cluster subunit redox states, the proximal cubane remains in oxidation²³ state II, [Fe₄-S₄]²⁺. Computational and experimental¹⁻³,⁶,⁷,¹³⁻¹⁵,¹⁹,²¹,²⁴⁻⁵² [Fe-Fe]-hydrogenase H-cluster (and synthetic H-cluster analogues) research corroborates the potential redox states of the di-iron H-cluster subunit, Feₚ-Feₐ, where Feₚ¹⁻Feₐ¹, EPR silent, is the reduced di-iron H-cluster subunit, FeₚⅡ⁻Feₐ¹, paramagnetic, is the partially oxidized, and catalytically active di-iron subunit, and FeₚⅡ⁻FeₐⅡ, EPR silent¹⁴,⁵³, is the fully oxidized, inactive biferrous subunit, and has an OH⁻ or H₂O molecule bound to the FeₐⅡ.

The di-iron atoms are named proximal and distal, Feₚ-Feₐ (for Feₚ is closest to the ‘proximal’ cubane, while Feₐ is ‘distal’ from the cubane).
Figure 1-4. The DdH H-cluster and its subclusters (modified from reference 6).
1.5. [Fe-Fe]-hydrogenases spectroscopic studies

By performing spectroscopic studies on [Fe-Fe]-hydrogenases which, have been purified from *Desulfovibrio desulfuricans* (Figure 1-5, 1-6) and *Clostridium pasteurianum* (Figure 1-7, 1-8, 1-9), 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\(_2\)O 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)\(^{12,14}\).
Figure 1-5. Crystal structure of *Desulfovibrio desulfuricans* hydrogenase, DdH\textsuperscript{17}. 
Figure 1-6. DdH prosthetic groups\textsuperscript{17}.
Figure 1-7. Crystal structure of Clostridium pasteurianum [Fe-Fe]-hydrogenase, CPI$^{15}$. 
Figure 1-8. CPI [Fe-Fe]-hydrogenase prosthetic groups\textsuperscript{15}.\vspace{1cm}
Figure 1-9. CPI [Fe-Fe]-hydrogenase [Fe₂S₂] and cubane subclusters\textsuperscript{15} (point group symmetry $= D_{2d}$, not an $O_h$).
This investigation is subdivided into three parts, viz., thermodynamics, geometric, and electronic analysis, for both wild-type and mutated (residue substituted, Figure 1-10) DdH. These analyses were carried out in order to understand the thermodynamic results, their relationship to certain molecular spatial behavior, e.g., CO\textsubscript{6} movement, and the electronic structural methods, such as frontier molecular orbitals (FMO), and natural bond orbital partial charges (NBO).
**Figure 1-10.** In the above DdH, an 8 Å residue red layer is studied concerning the potential inhibitory residues hindering H₂O elimination¹⁷.
References


CHAPTER II

REACTIVATION PATHWAY OF THE HYDROGENASE H-CLUSTER: A DENSITY FUNCTIONAL THEORY STUDY

2.1. Abstract

This paper puts forth a reaction pathway for the reactivation of exogenous ligand inhibited complex, Fe-Fe, with the metal centers bridged by di(thiomethyl)amine. Exogenous ligands, H₂O, and OH⁻, are bound to the distal iron (Fe₈). Density functional theory calculations on the native and ruthenium-modified H-cluster have been performed using the B3LYP functional with 6-31+G** and 6-311+G** bases sets. We have ascertained that there is a thermodynamically favorable pathway for the reactivation of the OH⁻ inhibited H-cluster, which proceeds by an initial protonation of
The Fe₆-OH⁻ complex. The proposed reaction pathway has all of its intermediate reactions ensue exothermically.

2.2. Introduction

Hydrogenases are a family of enzymes that reversibly catalyze the transformation of protons and electrons to molecular hydrogen (2H⁺ + 2e⁻ → H₂). The exploration for alternative energy sources has engendered great interest in hydrogenase research¹⁻⁶. However, these enzymes seem to be inactivated by exogenous ligands⁷, such as O₂, and OH⁻. Water also binds to the active site, in the resting state, of the enzyme⁸,⁹, Fe_p⁻²⁻Fe_d⁻²⁻ (where Fe_p is the proximal iron, and Fe_d is the distal iron).

The recently detailed structures of Fe-only hydrogenases, from Clostridium pasteurianum (CPI)¹⁰,¹¹ and Desulfövibrio desulfuricans (Dd)¹²,¹³, offer new opportunities for understanding their functions via biochemical pathways⁷,¹⁴. It is now possible to determine the inhibitory mechanisms of O₂, and OH⁻ by performing density functional theory (DFT) calculations on the active site of these enzymes, i.e., the H-cluster. This cluster is comprised of two iron atoms that are bridged by di(thiomethyl)amine (DTMA) group, and are coordinated by endogenous cyanide, carbon monoxide ligands, and a bridging carbonyl (COₖ). At the proximal metal, a cysteine-S bridging occurs to a [4Fe-4S] cubane, but in our investigation cysteine is replaced with CH₃-S, and the cubane is exchanged with H⁺ (Figure 2-1).
Figure 2-1. The H-cluster structure (M = Fe, Ru; L = exogenous ligand).
By performing DFT calculations on the H-cluster, with OH\textsuperscript{-}, and O\textsubscript{2} bound to Fe\textsubscript{d}, (redox states, Fe\texttextsuperscript{I}-Fe\texttextsuperscript{I}, Fe\texttextsuperscript{I}-Fe\texttextsuperscript{II}, Fe\texttextsuperscript{II}-Fe\texttextsuperscript{II}), Liu and Hu\textsuperscript{7} have inferred, based on agreement between the calculated and experimental vibrational frequencies of the three endogenous CO ligands, that OH\textsuperscript{-} is the oxygen species which inhibits hydrogenases.

2.3. Methods

The electronic structure of the H-cluster (Fe-only, and Ru-modified), was investigated by quantum mechanics (Gaussian-03\textsuperscript{15}), using the DFT method (B3LYP functional\textsuperscript{16,17}), with a variety of bases sets. Exploratory calculations have been performed with the 6-31+G(d, p) basis set, and further refined with 6-311+G(d, p) basis set. For Fe and Ru an effective-core potential with a double-zeta polarization basis set (LANL2DZ)\textsuperscript{18,19} was used.

In accordance with experimental and in-silico data, we selected low spin states (singlet, and doublet) and low oxidation states\textsuperscript{a} (I, and II) for the metal atoms\textsuperscript{20-25}.

Under the influence of a strong CN\textsuperscript{-} and CO crystal field on the d orbitals of the 2Fe subunit, the electric field induces large energy splitting between the e\textsubscript{g} and t\textsubscript{2g} orbitals. Electrons will then only fill the lower orbitals, giving an iron ground-state t\textsubscript{2g}\textsuperscript{6} (Fe\textsuperscript{II}) and t\textsubscript{2g}\textsuperscript{6}e\textsubscript{g}\textsuperscript{1} (Fe\textsuperscript{I}), with the H-clusters having low spin states.

\textsuperscript{a}Hall et al. have used low oxidation, Fe\textsuperscript{II}-Fe\textsuperscript{II}; Fe\textsuperscript{I}-Fe\textsuperscript{I}, (and spin states) rather than high oxidation states. Computational and experimental data show that the Fe\textsuperscript{I}-Fe\textsuperscript{I} species are the most stable complexes in the di-iron subcluster. In order to obtain Fe\textsuperscript{III}-Fe\textsuperscript{III} or Fe\textsuperscript{III}-Fe\textsuperscript{II}, electrons must be removed from the t\textsubscript{2g}-2\pi orbital, which is a stronger bonding orbital and it is not energetically feasible.
System stabilization of the Fe$^{1}$-Fe$^{1}$ subcluster is obtained by the fully occupied bonding orbital \([e_g-2\pi(\text{CO})]^{21,22}\) occurring between the Fe$_d$ and CO$_b$, which makes low oxidation states (Fe$^{II}$-Fe$^{II}$; Fe$^{II}$-Fe$^{1}$; Fe$^{1}$Fe$^{1}$) favorable. Note that a 4-electron oxidation from Fe$^{1}$-Fe$^{1}$ subunit is rather improbable.

2.4. Results and Discussion

Liu and Hu\textsuperscript{7} showed (reactivation$^6$ pathway I, Scheme 2-1) that Fe$_d^{II}$-OH$^{-}$ (1), can be further reduced to Fe$_d^{1}$-OH$^{-}$ (2)$\gamma$, but we found that the electron transfer is endothermic ($\Delta H = +12.31$ kcal/mol, Table 2-I) because the H-cluster (1) is already negatively charged (-1 e.u.). Fe$_d^{1}$-OH$^{-}$ (2) can be easily protonated to Fe$_d^{1}$-OH$_2$ (3) because its proton affinity is $+410.8$ kcal/mol. The water dissociation from Fe$_d^{1}$-OH$_2$ (3) is also endothermic ($\Delta H = +5.92$ kcal/mol).

Scheme 2 shows the reactivation pathway II in which the protonation step occurs first. This step proceeds because the proton affinity of Fe$_d^{II}$-OH$^{-}$ (1) is still very large ($+326.95$ kcal/mol, Table 2-II). Furthermore, Scheme 2-2 shows that water dissociation

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$^6$The process whereby the H-cluster is set free, once again, of exogenous ligands, i.e., OH$^{-}$, H$_2$O, etc., at the active site, Fe$_d^{1}$, in its reduced form. Hence in its reactivated state, the H-cluster is enabled to engage in its (former) catalytic H$_2$ redox activity.

$^7$There have been several source already cited corroborating the existence of standard DFT functionals and basis sets that are appropriate for modeling Fe$^{1}$ calibrations.\textsuperscript{7,14,21-24}

Also, by assessing the NBO charges of Fe$^{II}$ and Fe$^{1}$, small differences have been obtained for the iron species partial charges, i.e., 0.123 e.u. for compound 2, 0.106 e.u. for compound 3, and 0.102 e.u. for compound 4 (where these differences in partial charged have been obtained by means of absolute values).
from Fe$_d^{II}$-OH$_2$ (2') is also exothermic ($\Delta H = -3.22$ kcal/mol). In contrast, Cao and Hall$^{22}$ found that, the removal of water from the distal iron of Fe$_p^{II}$-Fe$_d^{III}$ is rather endothermic ($\Delta H = +23$ kcal/mol).
Scheme 2-1. Reactivation pathway I of Fe-only, and Ru-modified H-cluster.
Scheme 2-2. Reactivation pathway II of Fe-only, and Ru-modified H-cluster.
The difference in the reaction enthalpy calculated by Cao and Hall\textsuperscript{22}, relative to our result, may stem from the fact that the optimized structure, Fe\textsuperscript{II}\textsubscript{p}-Fe\textsuperscript{II}\textsubscript{d} (3'), in Cao and Hall’s study has the bridging carbonyl ligand (CO\textsubscript{b}) midway between Fe\textsubscript{p} and Fe\textsubscript{d}, which makes the H-cluster higher in energy than it is when CO\textsubscript{b} is bound (asymmetrically) closer to Fe\textsubscript{d} (Scheme 2-2, (3')). However, we were not able to find a stationary point (energy minimum) at B3LYP/6-31+G(d,p) level for the structure with symmetrically bound CO\textsubscript{b}, but we obtained a partially optimized structure by constraining only the distance between CO\textsubscript{b} and Fe\textsubscript{p} – the breaking bond – at 2.147 Å (the distance between CO\textsubscript{b} and Fe\textsubscript{d} was reduced from 2.040 Å to 1.873 Å during optimization). This quasi-symmetrical structure is 14 kcal mol\textsuperscript{-1} higher in energy than the structure with CO\textsubscript{b} bound asymmetrically (3') to the two irons. Thus a symmetrically CO\textsubscript{b} bound structure is expected to be even higher in energy. Hence, the removal of H\textsubscript{2}O (Scheme 2-2) is exothermic, for the CO\textsubscript{b} has been shifted towards Fe\textsubscript{d}, and this facilitates exogenous ligand bond breaking\textsuperscript{14}. Another structural detail contributing to the difference in the reaction enthalpies, 2' → 3', is that in the reactivation pathway of Scheme 2 (same for Liu and Hu\textsuperscript{7}), the non-bridging sulfur bound to Fe\textsubscript{p} is protonated. Also, due to different levels of theory that each investigating group had used, inevitably different optimized geometries are obtained.
Table 2-I. Reaction enthalpies for elementary reaction steps of the reactivation pathway I

<table>
<thead>
<tr>
<th>Metal combination</th>
<th>Reaction enthalpy$^a$</th>
<th>1 → 2</th>
<th>2 → 3</th>
<th>3 → 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-Fe</td>
<td>$+12.31^b$</td>
<td>-410.80</td>
<td>+5.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$+12.75^c$</td>
<td>-411.64</td>
<td>+5.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+10.02</td>
<td>-406.49</td>
<td>+3.42</td>
<td></td>
</tr>
<tr>
<td>Fe-Ru</td>
<td>+10.37</td>
<td>-406.92</td>
<td>+3.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+14.14</td>
<td>-413.96</td>
<td>+4.93</td>
<td></td>
</tr>
<tr>
<td>Ru-Fe</td>
<td>+14.51</td>
<td>-414.67</td>
<td>+4.89</td>
<td></td>
</tr>
<tr>
<td>Ru-Ru</td>
<td>+15.90</td>
<td>-412.58</td>
<td>+11.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+16.12</td>
<td>-412.93</td>
<td>+11.80</td>
<td></td>
</tr>
</tbody>
</table>

$^a$In kcal/mol.

$^b$Results obtained at B3LYP/6-31+G(d,p) level.

$^c$Results obtained at B3LYP/6-311+G(d,p) level.
Table 2-II. Reaction enthalpies for elementary reaction steps of the reactivation pathway II

<table>
<thead>
<tr>
<th>Metal combination</th>
<th>1 → 2'</th>
<th>2' → 3'</th>
<th>3' → 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-Fe</td>
<td>-326.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.22</td>
<td>-62.41</td>
</tr>
<tr>
<td></td>
<td>-327.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-3.19</td>
<td>-62.25</td>
</tr>
<tr>
<td>Fe-Ru</td>
<td>-331.14</td>
<td>+5.13</td>
<td>-67.02</td>
</tr>
<tr>
<td></td>
<td>-331.50</td>
<td>+4.96</td>
<td>-66.63</td>
</tr>
<tr>
<td>Ru-Fe</td>
<td>-329.56</td>
<td>+10.26</td>
<td>-75.59</td>
</tr>
<tr>
<td></td>
<td>-330.21</td>
<td>+10.46</td>
<td>-75.52</td>
</tr>
<tr>
<td>Ru-Ru</td>
<td>-328.13</td>
<td>+6.06</td>
<td>-62.67</td>
</tr>
<tr>
<td></td>
<td>-328.52</td>
<td>+5.89</td>
<td>-62.38</td>
</tr>
</tbody>
</table>

<sup>a</sup>In kcal/mol.

<sup>b</sup>Results obtained at B3LYP/6-31+G(d,p) level.

<sup>c</sup>Results obtained at B3LYP/6-311+G(d,p) level.
The result of this investigation, regarding water removal from Fe\textsubscript{d}\textsuperscript{II}, corroborates an antithetical reactivation pathway, that is, the reduction of Fe\textsubscript{p}\textsuperscript{II}-Fe\textsubscript{d}\textsuperscript{II} (3') is exothermic (unlike Scheme 2-1\textsuperscript{22}), for the CO\textsubscript{b} is only bound to Fe\textsubscript{d}\textsuperscript{II} and not to Fe\textsubscript{p}\textsuperscript{II}. Our results indicate that water removal from Fe\textsubscript{d}\textsuperscript{II} is facilitated by concerted bond breaking of CO\textsubscript{b} from Fe\textsubscript{p}\textsuperscript{II} and bond contraction between CO\textsubscript{b} and Fe\textsubscript{d}\textsuperscript{II} (as the bridging carbonyl migrates towards Fe\textsubscript{d}\textsuperscript{II}), Table 2-III. The reactivation of the H-cluseter is complete upon the reduction\textsuperscript{26} of Fe\textsubscript{d}\textsuperscript{II} to Fe\textsubscript{d}\textsuperscript{I}. This reduction process is highly exothermic ($\Delta H = -62.41$ kcal/mol, Table 2-II).

This reaction pathway (Scheme 2-2) thermodynamically favors the reactivation of the H-cluster, and suggests that the H-cluster may not be permanently inhibited by OH\textsuperscript{-}, or H\textsubscript{2}O, nonetheless. It seems that as long as the H-cluster is supplied protons, its reactivation shall continue.

The following bimetal combinations within the H-cluster, i.e., Fe\textsubscript{p}-Fe\textsubscript{d}, Fe\textsubscript{p}-Ru\textsubscript{d}, Ru\textsubscript{p}-Fe\textsubscript{d}, and Ru\textsubscript{p}-Ru\textsubscript{d}, were theoretically investigated (Table 2-I and 2-II) in order to elucidate which combinations are less sensitive to OH\textsuperscript{-} inhibition, and to find whether the varied metal combinations perform thermodynamically better than the Fe-Fe H-cluster. Table 2-I lists reaction enthalpies for the reactivation pathway I. For Fe\textsubscript{p}-Ru\textsubscript{d} modified H-cluster the electron transfer is slightly less endothermic (+2.29 kcal/mol) than for Fe\textsubscript{p}-Fe\textsubscript{d} H-cluster. The proton affinities for Ru\textsubscript{p}-Fe\textsubscript{d}, and Ru\textsubscript{p}-Ru\textsubscript{d} are slightly larger than for Fe-only, except Fe\textsubscript{p}-Ru\textsubscript{d}. However, in the H\textsubscript{2}O removal step, the bimets Fe\textsubscript{p}-Ru\textsubscript{d} and Ru\textsubscript{p}-Fe\textsubscript{d} release water more readily than the Fe-only cluster. Conversely, calculations on Ru-only H-cluster indicate that Ru\textsubscript{d} binds water more firmly than Fe\textsubscript{d} (Table 2-I).
**Table 2-III.** Bond lengths \((M_p\text{-CO}_b \text{ and } M_d\text{-CO}_b)\) for the reactant, product and intermediates that appear in reactivation pathways I and II of hydrogenase H-cluster

<table>
<thead>
<tr>
<th>Di-metals</th>
<th>Compound</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>2’</th>
<th>3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-Fe</td>
<td></td>
<td>2.341&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.817</td>
<td>2.013</td>
<td>2.232</td>
<td>2.411</td>
<td>3.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.829&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.807</td>
<td>1.945</td>
<td>1.850</td>
<td>1.845</td>
<td>1.819</td>
</tr>
<tr>
<td>Fe-Ru</td>
<td></td>
<td>2.541</td>
<td>2.935</td>
<td>2.161</td>
<td>3.710</td>
<td>2.661</td>
<td>3.250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.935</td>
<td>1.922</td>
<td>1.977</td>
<td>1.902</td>
<td>1.909</td>
<td>1.915</td>
</tr>
<tr>
<td>Ru-Fe</td>
<td></td>
<td>2.141</td>
<td>2.083</td>
<td>2.072</td>
<td>2.110</td>
<td>1.942</td>
<td>1.991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.961</td>
<td>2.005</td>
<td>1.984</td>
<td>1.947</td>
<td>2.503</td>
<td>2.487</td>
</tr>
<tr>
<td>Ru-Ru</td>
<td></td>
<td>2.458</td>
<td>2.245</td>
<td>2.159</td>
<td>2.170</td>
<td>2.609</td>
<td>3.362</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.966</td>
<td>2.032</td>
<td>2.019</td>
<td>2.009</td>
<td>1.930</td>
<td>1.918</td>
</tr>
</tbody>
</table>

<sup>a</sup>In Å.

<sup>b</sup>Results obtained at B3LYP/6-31+G(d,p) level for \(M_p\text{-CO}_c\).

<sup>c</sup>Results obtained at B3LYP/6-31+G(d,p) level for \(M_d\text{-CO}_c\).
On the other hand, for the reactivation pathway II, protonation of bimetal combinations, i.e., Fe\textsubscript{p}-Ru\textsubscript{d}, Ru\textsubscript{p}-Fe\textsubscript{d}, and Ru-only, is highly exothermic (similar to the Fe-only H-cluster). However, water removal is endothermic for these bimetal combinations. Subsequently, the reduction process (Scheme 2-2) necessitates similar enthalpies of reaction for most bimetal combinations (Table 2-II) mentioned above except for Ru\textsubscript{p}-Fe\textsubscript{d} which is slightly more exothermic.

2.5. Gibbs’ Energy Analysis

In pathway I, 1 \rightarrow 2, Gibbs’ free energy, for all bimetal combinations, is nonspontaneous (with the sign being positive as for the enthalpy calculations, Table 2-IV).

Antithetical to the above step, 2 \rightarrow 3, Gibbs’ free energy, for every bimetal combination, is highly exergonic (being negative as the former enthalpy calculations).

For pathway I, 3 \rightarrow 4, Gibbs’ free energy is exergonic (as opposed to the endothermic enthalpies).

However, in spite of the observed spontaneity for protonation and H\textsubscript{2}O removal, the overall pathway I is nevertheless hindered from being completed due to the reductive step, 1 \rightarrow 2.

In pathway II, 1 \rightarrow 2’, Gibbs’ free energy, for the studied bimetal combination, is rather exergonic (being negative as for the enthalpy calculations).
However, for $2' \rightarrow 3'$, Gibbs’ free energy is exergonic for all bimetal combinations (not just for the exothermic Fe-Fe species), fact which makes pathway II fully spontaneous.

Finally, for $3' \rightarrow 4$, the reaction is spontaneous for all bimetal combinations, (with the sign being negative as for the enthalpy calculations, Table 2-V).
Table 2-IV. Reaction entropies (T*ΔS), and free energies for the elementary reaction steps of the reactivation pathway I

<table>
<thead>
<tr>
<th>Bimetals</th>
<th>1 → 2 (+e⁻)</th>
<th>2 → 3 (+H⁺)</th>
<th>3 → 4 (-H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*ΔS</td>
<td>ΔG⁺</td>
<td>T*ΔS</td>
</tr>
<tr>
<td>Fe-Fe</td>
<td>+4.10</td>
<td>+8.21⁺</td>
<td>-1.63</td>
</tr>
<tr>
<td></td>
<td>+4.23</td>
<td>+8.52⁺</td>
<td>-1.76</td>
</tr>
<tr>
<td>Fe-Ru</td>
<td>+4.08</td>
<td>+5.94⁺</td>
<td>-1.34</td>
</tr>
<tr>
<td></td>
<td>+4.16</td>
<td>+6.21⁺</td>
<td>-1.44</td>
</tr>
<tr>
<td>Ru-Fe</td>
<td>+3.29</td>
<td>+10.85⁺</td>
<td>-0.56</td>
</tr>
<tr>
<td></td>
<td>+3.33</td>
<td>+11.18⁺</td>
<td>-0.50</td>
</tr>
<tr>
<td>Ru-Ru</td>
<td>+2.50</td>
<td>+13.4⁺</td>
<td>-0.39</td>
</tr>
<tr>
<td></td>
<td>+2.55</td>
<td>+13.57⁺</td>
<td>-0.43</td>
</tr>
</tbody>
</table>

*In kcal/mol.

⁺Results obtained at B3LYP/6-31+G(d,p) level.

⁺⁺Results obtained at B3LYP/6-311+G(d,p) level.
**Table 2-V.** Reaction entropies (T*ΔS), and free energies for the elementary reaction steps of the reactivation pathway II

<table>
<thead>
<tr>
<th>Bimetals</th>
<th>1 → 2' (+H'&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>2' → 3' (-H&lt;sub&gt;2&lt;/sub&gt;O)</th>
<th>3 → 4 (+e&lt;sup&gt;-&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*ΔS</td>
<td>ΔG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T*ΔS</td>
</tr>
<tr>
<td>Fe-Fe</td>
<td>+1.36</td>
<td>-328.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+13.33</td>
</tr>
<tr>
<td></td>
<td>+1.56</td>
<td>-329.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+13.23</td>
</tr>
<tr>
<td>Fe-Ru</td>
<td>+1.15</td>
<td>-332.29</td>
<td>+13.28</td>
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<tr>
<td></td>
<td>+1.09</td>
<td>-332.59</td>
<td>+13.14</td>
</tr>
<tr>
<td>Ru-Fe</td>
<td>+1.33</td>
<td>-330.89</td>
<td>+13.66</td>
</tr>
<tr>
<td></td>
<td>+1.37</td>
<td>-331.58</td>
<td>+13.57</td>
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<tr>
<td>Ru-Ru</td>
<td>+1.23</td>
<td>-329.36</td>
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</tr>
<tr>
<td></td>
<td>+1.08</td>
<td>-329.60</td>
<td>+13.27</td>
</tr>
</tbody>
</table>

<sup>a</sup>In kcal/mol.

<sup>b</sup>Results obtained at B3LYP/6-31+G(d,p) level.

<sup>c</sup>Results obtained at B3LYP/6-311+G(d,p) level.
2.6. Conclusions

Reactivation pathway I consists of an endothermic electron transfer step, followed by an exothermic protonation step, and then an endothermic water removal step. For reactivation pathway II, the H-cluster protonation occurs first, followed by water removal, and then by electron transfer with all steps being exothermic.

Finally, we propose a reaction pathway for the reactivation of H-cluster, i.e., pathway II. Hence, this H-cluster reaction pathway has the potential of functioning aerobically.

Crystallographic data of *Clostridium Pasteurianum* Fe-only hydrogenase shows that the H-cluster is inhibited in the air when (either) OH (or H₂O) is bound to the distal iron, Fe₁Ⅱ. The reason the OH has been chosen as the H-cluster’s inhibiting species is due to the closeness of the experimental to the theoretical vibrational spectra. Thus, “the potential of functioning aerobically” denotes that because the individual reactions in Scheme 2 are all exothermic, the H-cluster does become reactivated, hence it is able to produce hydrogen gas [(by reduction of protons) if electrochemists should decide to spray H-clusters on electrodes, thus replacing the more expensive platinum catalyst].
References


CHAPTER III
REACTION PATHWAYS, IN AQUEOUS PHASE, FOR THE
REACTIVATION OF THE EXOGENOUSLY INHIBITED [FE-FE]-
HYDROGENASES

3.1. The ONIOM Method

The subsequent investigation deals with the inhibition mechanism of H-cluster\(^1\)\(^{-}49\) by OH\(^-\), which uses the hybrid ONIOM\(^a\) (MM/QM) method on the enzyme matrix.

\(^a\)ONIOM (Acronym: Our owN n-layered Integrated molecular Orbital + molecular mechanics Method), as in our study, consists of two layers. One is the QM and the other the MM. The QM level treats the [Fe-Fe]-hydrogenase H-cluster, and the MM level handles the remaining enzyme-solvent environment. The QM/MM method calculates both the localized active site, [Fe-Fe]-hydrogenase H-cluster, and the rest of the enzyme.
Gromacs software\textsuperscript{50,51} is made use of to add hydrogen atoms, water, and counter ions to the X-ray crystal structure [of the Brookhaven Protein Data Bank (id.1HFE)] of DdH\textsuperscript{b}. The crystal structure missing hydrogen atoms and H\textsubscript{2}O molecules (of 1 nm layer) are incorporated into the studied PDB DdH structure, while the sodium cations (6 - 8) are arbitrarily introduced into the DdH structure in order to render the structure neutral.

For the ONIOM optimizations carried on the [Fe-Fe]-hydrogenase, two layers had been used. One is the low layer, which is comprised of the hydrogenase amino acid residues, and three cubanes (i.e., proximal, medial, and distal), to which the MM\textsuperscript{52-58} method is applied. The other is the high layer, [Fe-Fe]-hydrogenase H-cluster, for which the QM method is used to calculate the electronic and geometric properties. Specifically, the high layer contains a 2Fe subunit, Fe\textsubscript{p}-Fe\textsubscript{d}, constituting the H-cluster, S\textsubscript{γ}, and C\textsubscript{β} which belong to the bridging Cys\textsuperscript{382}. Moreover, two linking atoms, i.e., hydrogen atoms, are added between the following atoms. A linking hydrogen is connected between C\textsubscript{α} and C\textsubscript{β}, and the other between S\textsubscript{γ} (of Cys\textsuperscript{382}) and Fe (where the latter atom belongs to the proximal cubane). Also, the ONIOM method is a hybrid QM/MM method. [The computational QM methods had been described above (Chapter II)]. The MM method\textsuperscript{c}

\textsuperscript{b}Counter ions are added to simulate the surrounding environment of enzymes.

\textsuperscript{c}Molecular mechanics makes use of Newtonian mechanics for the purpose of modeling molecular systems. MM (ignores electrons) is based on a model of molecules as a collection of atoms (balls) held together by bonds (springs). By changing the molecular geometry (the shapes of molecules, i.e., bond lengths, angles, and dihedrals) until the lowest energy is obtained, one finds the optimized geometry.
employs the universal force field\textsuperscript{59} (UFF\textsuperscript{5}), and the charge equilibration method\textsuperscript{6} for Coulomb interactions. The hydrogen bonds, within the H-domain, which are encountered between the H-cluster, and the proximal (key) amino acids, are examined making use of Chem3D, and Pymol programs\textsuperscript{60,61}.

\textsuperscript{5}A force field consists of several potential energy functions which describe various interactions, such as bond stretching, angle bending, dihedral torsion, and pair, non-bonded electrostatic interactions, and their parameters help describe the potential energy for a system of particles.

\textsuperscript{6}The charge equilibration method (QEq) allows charges respond to applied fields, or to changes in the system.
Figure 3-1. The QM/MM (ONIOM) Method for the Enzyme System. The active center is treated at the QM Level, and the remainder is treated at the MM level.
3.2. Results and Discussion

The subsequent investigation addresses the catabolic reactivation mechanism of the hydrogenase H-cluster by OH⁻ removal, which uses the ONIOM method for the studied mechanism.

For ONIOM calculations, Scheme 2-1, 1 → 2, of [Fe-Fe]-hydrogenase H-cluster, the reductive process ensues slightly endothermically ($\Delta H_{\text{Enzyme}}^\theta = +2.08 \text{ kcal/mol}$, Table 3-I). The enzyme matrix calculations, therefore, 1 → 2, relative to those of the gas phase ($\Delta H_{\text{Gas}}^\theta = +12.31 \text{ kcal/mol}$, Table 2-I), are less endothermic by a difference of +10.23 kcal/mol, which points out that DdH undergoes this reductive, heat of reaction process better than in gas phase.

---

Where $\Phi$ means phase.
**Table 3-I.** Reaction enthalpies for elementary steps of the reactivation pathways I and II

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Reaction 1 → 2</th>
<th>Reaction 2 → 3</th>
<th>Reaction 3 → 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen Enzyme ( \Phi )</td>
<td>+3.33</td>
<td>-210.13</td>
<td>+8.92</td>
</tr>
<tr>
<td>Enzyme ( \Phi )</td>
<td>+2.08</td>
<td>-213.03</td>
<td>+25.96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Reaction 1 → 2’</th>
<th>Reaction 2’ → 3’</th>
<th>Reaction 3’ → 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen Enzyme ( \Phi )</td>
<td>-132.52</td>
<td>+24.89</td>
<td>-90.24</td>
</tr>
<tr>
<td>Enzyme ( \Phi )</td>
<td>-130.37</td>
<td>+19.60</td>
<td>-74.22</td>
</tr>
</tbody>
</table>

\(^{a}\)Results obtained at B3LYP/6-31+G(d,p) level, in kcal/mol.

---

\(^{a}\)A “frozen” enzyme method, used for QM/MM setup (in ONIOM calculations), is less accurate because the Cartesian coordinates of the selected atoms are “fixed” in space, hence giving less reliable results (but has advantages in acquiring preliminary results, and lessening the computational time).
For the [Fe-Fe]-hydrogenase H-cluster oxidation result, 2 → 3, a highly exothermic reaction is obtained ($\Delta H_{\text{Enzyme}} = -213.03$ kcal/mol). Comparing the oxidation, 2 → 3, of the two environments, i.e., the entire hydrogenase H-cluster with the H-cluster in vacuum, the obtained enthalpy difference is less (by about half, $\Delta H = +197.77$ kcal/mol) for the enzyme matrix, which does make sense biologically (i.e., if the heat of reaction were too high, then perforce enzymatic denaturation would result).

In 3 → 4, the [Fe-Fe]-hydrogenase H-cluster calculations, for H$_2$O elimination, show the most endothermic reaction ($\Delta H_{\text{Enzyme}} = +25.96$ kcal/mol) in its own class (i.e., of both Scheme 2-1 and 2-2).

Comparing $\Delta H_{\text{Enzyme}} = +25.96$ kcal/mol with $\Delta H_{\text{Gas}} = +5.92$ kcal/mol, the H$_2$O removal is more difficult for DdH due to the positive charges of the quaternary ammonium ions, -NH$_3^+$, from Lys$^{237}$ and Lys$^{238}$ which repel the positive charge of the water ($\delta^+\text{H}_2\text{O}, \text{Enzyme} = 0.087$ a.u., Fe$_d$–H$_2$O$^+$), although the Fe$_d$’s Natural Bond Orbital [(partial charges), NBO] charge is only -0.051 a.u. (Figure 3-6); whereas in gas phase, such repelling positive charges are absent.

For Scheme 2-2, 1 → 2’, the [Fe-Fe]-hydrogenase H-cluster oxidation provides an exothermic reaction ($\Delta H_{\text{Enzyme}} = -130.37$ kcal/mol); its oxidation enthalpy is considerably less than in gas phase ($\Delta H_{\text{Gas}} = -326.95$ kcal/mol), correlating the trend in Scheme 1.

In 2’ → 3’, the enzyme $\Phi$ calculations, for the H$_2$O removal step, confer an endothermic result ($\Delta H_{\text{Enzyme}} = +19.60$ kcal/mol). Comparing the DdH calculations to those of the gas $\Phi$ ($\Delta H_{\text{Gas}} = -3.22$ kcal/mol), a difference ($\Delta H_{\Phi \text{dif.}}$) of +22.82 kcal/mol between the studied phases is obtained. As opposed to the exothermic gas $\Phi$ step, the
reaction for the aqueous enzyme Φ occurs endothermically (ΔH_{Enzyme Φ} = +19.60 kcal/mol).

Hence, the water removal step is hindered (in the hydrogenase reactivation) due to the positive charges encountered on the quaternary ammonium ions of Lys^{237} and Lys^{238} which reside in the juxtaposed enzyme matrix, as mentioned in Scheme 2-1, 3 → 4.

Finally, for 3' → 4 (Scheme 2-2), the enzymatic aqueous Φ reduction step proceeds exothermically (ΔH_{Enzyme Φ} = -74.22 kcal/mol), just as in gas phase (ΔH_{Gas Φ} = -62.41 kcal/mol).

Therefore, the reactivation of [Fe-Fe]-hydrogenase H-cluster 4 (Scheme 2-2) could have been obtained, were it not for endothermic H\(_2\)O removal step, 2' → 3'.

### 3.3. Spatial Considerations

In order to better explain the enthalpy differences between the aqueous enzyme Φ and the gas Φ calculations, the [Fe-Fe]-hydrogenase H-cluster and H-cluster geometry for the two phases are analyzed, and then the hydrogenase H-cluster distances to the juxtaposed amino acids are presented for the aqueous enzyme Φ.

For the ONIOM calculations, the exogenous ligand, H\(_2\)O, removal is somewhat ambiguous regarding CO\(_b\) translation towards Fe\(_d\). That is, in Scheme 1', 3 → 4, the Fe\(_d\)-

\[\text{\textsuperscript{i}In the gas Φ, both Scheme 1 and 2 for H}_2\text{O removal show a concerted bond elongation between Fe}_p\text{-CO}\text{, and a bond contraction between Fe}_d\text{-CO}_b\text{, as seen in 3 → 4, and 2'} → 3' for Schemes 1, and 2, respectively. Hence, the shifting of the CO}_b\text{ towards Fe}_d\text{ seems to facilitate the exogenous ligand removal, e.g., H}_2\text{O, and H}_2^{18,44}.\]
CO\textsubscript{b} distance becomes longer (as opposed to the gas \( \Phi \) CO\textsubscript{b} migration, Table 3-II), which may account for one of the reasons why H\textsubscript{2}O removal is endothermic. Thus, it is ascertained that for the aqueous enzyme \( \Phi \), a decrease for the exogenous ligand removal is encountered upon Fe\textsubscript{d}-CO\textsubscript{b} bond elongation (agreeing with the gas \( \Phi \) Fe\textsubscript{d}-CO\textsubscript{b} bond contraction correlating directly to enhanced H\textsubscript{2}O elimination).
Table 3-II. The obtained bond lengths (Fe\(_p\)-CO\(_b\), and Fe\(_d\)-CO\(_b\)) for reactant 1, product 4, and intermediates 2, 2', 3, and 3' in the reactivation of Scheme 1 and 2 for the hydrogenase H-cluster.

<table>
<thead>
<tr>
<th>Distances (Å)</th>
<th>H-clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Fe(_p)-CO(_b)</td>
<td>2.554(^a)</td>
</tr>
<tr>
<td>Fe(_d)-CO(_b)</td>
<td>1.815</td>
</tr>
<tr>
<td>Fe(_p)-CO(_b)</td>
<td>2.341(^b)</td>
</tr>
<tr>
<td>Fe(_d)-CO(_b)</td>
<td>1.829</td>
</tr>
</tbody>
</table>

\(^a\)Obtained from ONIOM calculations.  
\(^b\)Obtained from gas Φ calculations.
Due to the distance (3.718 Å) between the distal iron and the proximal lysine, (Fe\textsubscript{d}…H•Lys\textsuperscript{237}), the latter may be a potential H\textsuperscript{+} donor to the Fe\textsubscript{d} according the modified (hydrogen addition) crystal structure (PDB ID: 1HFE)\textsuperscript{22}, as opposed to the ONIOM calculations, i.e., average distance\textsubscript{(Fe\textsubscript{d}…H•Lys\textsuperscript{237})} = 4.200 Å (Table 3-III)\textsuperscript{k}.

Because the crystal structure is a mere snapshot of interatomic distances, and suggests, therefore, that Fe\textsubscript{d} protonation may occur due to the Lys\textsuperscript{237}’s H\textsuperscript{+}, this does not seem to be the case according to our ONIOM calculations, for all the distances, Fe\textsubscript{d}…H•Lys\textsuperscript{237}, are larger. Hence, Fe\textsubscript{d} protonation may occur from the DTMA•N bridge protonation\textsuperscript{h}, or from some other surrounding amino acids.

According to both enhanced x-ray crystal structure (by Gromacs’ hydrogen addition) and ONIOM calculations for the interatomic (dotted) distances, DTMA•N…S•Cys\textsuperscript{178}, cysteine (pK\textsubscript{a} = 8.33) seems to (preliminarily) be one of the most likely candidates responsible for N\textsubscript{DTMA} protonation (Table 3-III). That is, with the nitrogen—gamma hydrogen bond (DTMA•N…H\textsubscript{\gamma}•Cys\textsuperscript{178}) varying to within normal N…H\textsubscript{\gamma} bond range {up to 2.42 Å [i.e., 3.74 Å (N…S) – 1.32 Å (S…H\textsubscript{\gamma}) = 2.42 Å]}, the potential protonation ability of Cys\textsuperscript{178} ought to be further researched using molecular dynamics.

Although the bond distance between N\textsubscript{DTMA} and H\textsuperscript{+} (sulfonium ion), DTMA•N…H\textsubscript{\delta}S\textsubscript{\delta}\textsuperscript{+}•Met\textsuperscript{376}, can get to within the normal bond range of about 2.5 Å

\textsuperscript{k}The H-bond (between N…H) in Fe\textsubscript{d}•CN…H•Lys\textsuperscript{237} is also longer than in the original crystal structure.

\textsuperscript{h}Which of the two potential protonation sources are most involved shall be shown by means of molecular dynamics.
(according to 3-D ONIOM results, Table 3-III), there is virtually no chance of proton transfer occurring because of the rather high (calculated) \( pK_a \) for \( \text{H}_5\text{S}_8^+\cdot\text{Met}^{376} \) (\( pK_a = 27.432 \)).

[The (dotted) distance, DTMA\cdot\text{N}…\text{H}_5\text{S}_8^+\cdot\text{Met}^{376}, was approximated by first measuring the distance from \( \text{N}_{\text{DTMA}} \) to \( \text{S}_8^+ \), then subtracting the \( \text{H}_5…\text{S}_8^+ \) bond distance from DTMA\cdot\text{N}…\text{S}_8^+\cdot\text{Met}^{376} \) bond length, i.e., 3.771 Å - 1.230 Å = 2.541 Å (Table 6).]

Additionally, the distances between Fe\(_d\)…\( \cdot\text{H}\cdot\text{Ser}^{202} \) and Fe\(_d\)…\( \cdot\text{H}\cdot\text{Thr}^{145} \) are exceedingly large in both the crystal structure as well as in the ONIOM calculations, thus the \( \cdot\text{H}^+ \) donating ability of Ser\(^{202}\) and Thr\(^{145}\) seems to be limited relative to that of Cys\(^{178}\) (Table 3-III).
Table 3-III. Bond lengths (Fe\textsubscript{p}-CO\textsubscript{b}; Fe\textsubscript{d}-CO\textsubscript{b}), and interatomic distances between the H-cluster and the juxtaposed key residues

<table>
<thead>
<tr>
<th></th>
<th>Original\textsuperscript{a} (Å)</th>
<th>(1)\textsuperscript{b}</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>2'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe\textsubscript{d}…H\textbullet Lys\textsuperscript{237/c}</td>
<td>3.718</td>
<td>4.249</td>
<td>4.229</td>
<td>4.173</td>
<td>4.161</td>
<td>4.157</td>
<td>4.208</td>
</tr>
<tr>
<td>Fe\textsubscript{d}•CN…H•Lys\textsuperscript{237}</td>
<td>2.162</td>
<td>2.720</td>
<td>2.690</td>
<td>2.694</td>
<td>2.633</td>
<td>2.667</td>
<td>2.726</td>
</tr>
<tr>
<td>Fe\textsubscript{d}…H•Ser\textsuperscript{202}</td>
<td>6.210</td>
<td>7.083</td>
<td>6.877</td>
<td>6.903</td>
<td>6.888</td>
<td>6.922</td>
<td>7.103</td>
</tr>
<tr>
<td>Fe\textsubscript{d}…H•Thr\textsuperscript{145}</td>
<td>7.048</td>
<td>7.309</td>
<td>7.316</td>
<td>7.297</td>
<td>7.053</td>
<td>7.114</td>
<td>7.192</td>
</tr>
<tr>
<td>DTMA•N…S•Cys\textsuperscript{178}</td>
<td>3.239</td>
<td>3.607</td>
<td>3.742</td>
<td>3.753</td>
<td>3.531</td>
<td>3.791</td>
<td>3.672</td>
</tr>
<tr>
<td>DTMA•N…H•Cys\textsuperscript{178}</td>
<td>2.905</td>
<td>4.376</td>
<td>4.451</td>
<td>4.41</td>
<td>3.657</td>
<td>4.166</td>
<td>3.805</td>
</tr>
<tr>
<td>DTMA•C…S•Met\textsuperscript{376}</td>
<td>3.815</td>
<td>3.858</td>
<td>3.773</td>
<td>3.975</td>
<td>3.921</td>
<td>4.076</td>
<td>3.986</td>
</tr>
<tr>
<td>DTMA•N…S•Met\textsuperscript{376}</td>
<td>3.900</td>
<td>3.794</td>
<td>3.771</td>
<td>4.009</td>
<td>4.004</td>
<td>4.178</td>
<td>3.903</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) The original PDB crystal structure.

\(\textsuperscript{b}\) Obtained from ONIOM calculations.

\(\textsuperscript{c}\) Where the “•” in H•Lys\textsuperscript{237} means that H belongs to the Lysine\textsuperscript{237}; the same applies for the other dotted species.
3.4. Frontier Molecular Orbital Analysis

Molecular orbital analysis is presented using frontier orbitals (HOMO, LUMO, and SOMO) in correlation with the formerly presented reaction enthalpies. Basically, for the reduction of open-shell H-clusters, one finds that an $e^-$ is transferred into an unoccupied molecular orbital, SOMO$_β$ (for SOMO$_α$ is occupied), while for the closed-shell clusters, an $e^-$ is transferred into the lowest unoccupied molecular orbital, LUMO. In the case of open-shell H-cluster oxidation (both gas and enzyme liquid $\Phi$), a $\sigma$-bond is formed by the interaction of electrons in the highest occupied molecular orbital (HOMO), or by the contributions of both HOMO and SOMO, given that the SOMO is close in energy relative to HOMO. Conversely, when a H$^+$ is in close proximity to a closed-shell cluster, the resulting $\sigma$-bond is mainly due to the contribution of $e^-$s from HOMO and the H$^+$. 
Figure 3-2. The NBO charges obtained (from $\alpha$ electrons) from DFT calculations (B3LYP/6-31+G(d,p) with LANL2DZ pseudo potential for Fe atoms). 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.
[Fe-Fe]-hydrogenase H-cluster 1, (Scheme 1, 1 → 2) becomes reduced, and according to the LUMO depiction, the $e^-$ should mainly be localized in the vicinity of the two irons, Fe$_p$-Fe$_d$. However, according to the NBO partial charge results (Figure 6), the irons (of hydrogenase H-cluster 1) do not have much affinity for the approaching $e^-$, i.e., $q_{Fe_p} = -0.043$ a.u., and $q_{Fe_d} = -0.099$ a.u. This, in turn, corroborates the endothermic enthalpy result ($\Delta H_{\text{Enzyme } \Phi} = + 2.08$ kcal/mol) for the cluster reduction.

Regarding [Fe-Fe]-hydrogenase H-cluster 2, (2 → 3), the HOMO$_\alpha$ has more orbital diffusion than HOMO$_\beta$ over the OH$^-$ (the following orbitals), hence, in conjunction with the NBO partial charge of the hydroxyl, $q_{OH^-} = -0.543$ a.u., the large H$^+$ affinity for cluster 2, $\Delta H = -213.03$ kcal/mol, (2 → 3) comes as no surprise. Moreover, comparing the relative orbital diffusions over the OH$^-$ of SOMO$_\alpha$ and HOMO$_\alpha$, it seems that the HO-H$^+$ $\sigma$-bond results from the combination of the HOMO$_\alpha$, HOMO$_\beta$, and SOMO$_\alpha$.
In H-cluster 3, (3 → 4), HOMO$_{\alpha}$, HOMO$_{\beta}$, and SOMO$_{\alpha}$ are diffused throughout the cluster except over the Fe$_d$-OH$_2$ bond, implying that the e's of the σ-bond, Fe$_d$-OH$_2$, reside in a lower energy state (HOMO -1), which also explains the negative NBO partial charges of both Fe$_d$ ($q_{Fe_d} = -0.325$ a.u.), and the O ($q_O = -0.503$ a.u.) of H$_2$O. This fact, therefore, may explain the tenacity of Fe$_d$ for H$_2$O, i.e., $\Delta H = +25.96$ kcal/mol.

For [Fe-Fe]-hydrogenase H-cluster 1, (Scheme 2-2, 1 → 2'), the HOMO is diffused over the central portion of the cluster ([Fe-Fe], and DTMA bridge) and over the exogenous ligand, i.e., OH'. In spite of the greater e' orbital diffusion over the DTMA bridge, the H$^+$ becomes captured by OH' for it is a stronger base than the N of the DTMA bridge, and its NBO charge (q) is higher than that of the N ($q_{OH'} = -1.054 > q_{N} = -0.734$). Also, these analyses do agree with the calculated hydrogenase H-cluster proton affinity, i.e., $\Delta H = -130.37$ kcal/mol.
For H-cluster 2¢, (Scheme 2-2, 2¢ → 3¢), the HOMO is diffused over the left of the molecule (around Feₚ) which means that the e’s of the σ-bond, Fe_d-OH₂, are situated in a lower energy state (HOMO -1), which also explains the negative NBO partial charges of both O (qₒ = -0.968 a.u.), and the Fe_d (qFed = -0.051 a.u.) of H₂O. Therefore, this accounts for the bond strength of Fe_d for H₂O, i.e., ΔH = +19.60 kcal/mol.

In addition, from geometrical considerations, it can be seen that the CO_b is closer to the Fe_d than in H-cluster 3, (Scheme 2-1, 3 → 4), which partially accounts for the less endothermic H₂O elimination process.

When H-cluster 3′, (3′ → 4) becomes reduced, the LUMO depiction shows whither the e’ may transfer, i.e., either to Feₚ or Fe_d. Then according to the NBO partial charge results, i.e., qFₚ = 0.048 a.u., and qFed = 0.046 a.u., in conjunction with the adjacent LUMO depiction, one can observe why the cluster reduction occurs with a rather high heat of reaction, i.e., ΔH_{EnzymeΦ} = -74.22 kcal/mol.
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CHAPTER IV

[Fe-Fe]-HYDROGENASE REACTIVATED BY RESIDUE MUTATIONS AS BRIDGING CARBONYL REARRANGES: A QM/MM STUDY

4.1. Abstract

The work presented here aims to find reaction pathways, in aqueous enzyme phase, for the reactivation of the exogenously inhibited [Fe-Fe]-hydrogenases, viz., by O₂, or OH⁻, which metabolizes to H₂O₁². The current investigation uses the hybrid quantum mechanics/molecular mechanics (QM/MM) method to study reactivation pathways for the exogenously inhibited enzyme matrix. ONIOM calculations performed on the enzyme agree with experimental results, i.e., the hydrogenase H-cluster is inhibited by oxygen metabolites. To investigate potential inhibitory residues that prevent H₂O from leaving the catalytic site, and reactivate the hydrogenase H-cluster, an enzyme spherical region of
radius 8 Å (from the distal iron, Fe₈, of [Fe-Fe]-hydrogenase H-cluster) was screened. In the screening process, polar residues were removed, one at a time, and frequency calculations provided the change in the Gibbs’ energy of water dissociation (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 of H₂O elimination. Favorable thermodynamic results have been obtained for both single residue removal (ΔGΔG_{Glu^{374}} = -1.6 kcal/mol), single substitution (ΔGΔG_{Glu^{374}His} = -3.1 kcal/mol), and combined residue substitutions (ΔGΔG_{Arg^{111}Glu; Thr^{145}Val; Glu^{374}His; Tyr^{375}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 more spontaneously. Thus, the most promising residue deletions which enhance H₂O elimination are ΔArg^{111}, ΔThr^{145}, ΔSer^{177}, ΔGlu^{240}, ΔGlu^{374}, and ΔTyr^{375}. Hence, both single and combined residue substituted [Fe-Fe]-hydrogenase show increased spontaneity for H₂O removal. The thermodynamics and electronic structure analyses show that CO₆ plays a concomitant role in the enzyme inhibition/reactivation. In gas phase, CO₆ shifts towards Fe₈ 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 CO₆ towards Fe₈, which leads to enzyme inhibition. But enzyme reactivation can be achieved by suitable amino acid substitutions.
4.2. Introduction

[Fe-Fe]- and [Ni-Fe]-hydrogenases\textsuperscript{3-123} are two major classes of enzymes that reversibly catalyze the apparently simple reaction of protons and electrons to molecular hydrogen, $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$, which occurs in anaerobic media. In living systems, of the two metalloproteins, [Fe-Fe]-hydrogenases are mostly used for $\text{H}_2$ 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 $\text{H}_2$ for the metabolic processes of these life forms. By means of $\text{H}_2$ oxidation, ATP synthesis exploits $\text{H}_2$ as an energy source, whereas $\text{H}_2$ synthesis results from the metabolic disposal of excess electron (with available protons), or from pyruvate fermentation. Proteins, such as ferredoxins, cytochrome c3, and cytochrome c6, act as physiological $\text{e}^-$ donors or acceptors\textsuperscript{81}. The exploration of alternative energy sources has kindled great interest in hydrogenase research. The reason for studying biological $\text{H}_2$ production is to clarify the complex mechanism (for hydrogen synthesis), which may help researchers produce clean fuel\textsuperscript{48}, using certain anaerobic organisms.

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

\textsuperscript{4}In fuel cells, the product, $(\text{H}_2 + \frac{1}{2}\text{O}_2 \rightarrow) \text{H}_2\text{O}$, is benign relative to the current, carcinogenic hydrocarbon combustion emissions.
By performing Density Functional Theory (DFT) calculations on the H-cluster, with H$_2$O, OH$^-$, and O$_2$ bound to Fe$_d$, (redox states Fe$^{II}$-Fe$^{II}$), Liu and Hu$^3$ 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 *Desulfovibrio desulfuricans* (Dd)$^6$ and *Clostridium pasteurianum* (CP1)$^7$, can be used to theoretically investigate their functions via biochemical pathways. Since former DFT, and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations$^{1-3,27}$ 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$^{1-3}$, CH$_3$-S$^-$ has been substituted for cysteine, Cys$^{382}$, and H$^+$ for the proximal cubane.$^6$

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$^6$A H$^+$ is replaced for [Fe$_4$S$_4$]$^{2+}$, and CH$_3$-S$^-$ for cysteine-S$^-$ in order to minimize computational time, and cost.
Figure 4-1. The H-cluster structure.
The active site of hydrogenases, viz., the H-cluster (Figure 4-1), is comprised of a dimetal complex, [Fe-Fe], with the metal centers being bridged by di(thiomethyl)amine (DTMA), 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.), and a bridging carbonyl (CO_b). 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 cysteiny1 sulfur (or S_γ of Cys^{382}).

In spite of the di-iron H-cluster subunit redox states, the proximal cubane remains in oxidation\(^8\) state II, \([\text{Fe}_4\text{-S}_4]^{2+}_p\). Computational and experimental\(^1\)-\(^5\) [Fe-Fe]-hydrogenase H-cluster (and synthetic H-cluster analogues) research corroborates the potential redox states of the di-iron H-cluster subunit, \([\text{Fe}_p\text{-Fe}_d]\), where \([\text{Fe}_p^{1}\text{-Fe}_d^{1}]\), EPR silent, is the reduced di-iron H-cluster subunit, \([\text{Fe}_p^{II}\text{-Fe}_d^{I}]\), paramagnetic, is the partially oxidized, and catalytically active di-iron subunit, and \([\text{Fe}_p^{II}\text{-Fe}_d^{II}]\), EPR silent\(^{113,125}\), is the fully oxidized, inactive 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\(^97,104,116\). 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^{118,141}. 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)\(^6,7\).

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\(^7\)The di-iron atoms are named proximal and distal, \([\text{Fe}_p\text{-Fe}_d]\) (for \(\text{Fe}_p\) is closest to the ‘proximal’ cubane, while \(\text{Fe}_d\) is ‘distal’ from the cubane).
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\textsubscript{b} movement, and the electronic structural methods, such as frontier molecular orbitals (FMO), and natural bond orbital partial charges (NBO).

4.3. Methods

The ONIOM method\textsuperscript{124,125} (DFT for the QM region, and the universal force field, UFF\textsuperscript{126}, for the MM region, implemented in Gaussian03\textsuperscript{127}) 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\textsuperscript{3} 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\textsuperscript{128,129}, and 6-31+G(d,p) basis set.

Hydrogen atoms were added to the X-ray crystal structure of DdH using the Gromacs\textsuperscript{130,131} [Brookhaven Protein Data Bank id.1HFE\textsuperscript{6}]. After the structure has been solvated [2043 H\textsubscript{2}O molecules (in 1 nm layer peripheral to H-cluster)], six Na\textsuperscript{+} ions were randomly incorporated into the solvent (7 Na\textsuperscript{+} ions for clusters 1, 3, 4; 8 Na\textsuperscript{+} ions for cluster 2) to neutralize the negative charges on the H-cluster, medial, and distal
cubane/cysteines clusters\textsuperscript{\textdegree}. 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\textsubscript{p} and CO\textsubscript{t,p}, in the QM region have been kept frozen\textsuperscript{\textdegree}. The rationale for freezing Fe\textsubscript{p}, and CO\textsubscript{t,p} arises from former optimizations\textsuperscript{\textdegree} 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. Frequency calculations treat both the apoenzyme and the cubanes as partial charges, whereas [Fe\textsubscript{p}-Fe\textsubscript{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\textsubscript{p}-Fe\textsubscript{d}] subunit, (the moiety of H-cluster), and C\textsubscript{β}, and S\textsubscript{γ} (cysteinyl sulfur of Cys\textsuperscript{382}). In order to avoid dangling bonds

\textsuperscript{\textdegree}Each of the three cubane/cysteine moieties (found in DdH) is comprised of a cubane plus four surrounding, depotonalated cysteines which are bound to the four iron atoms of every cubane.

\textsuperscript{\textdegree}A ‘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.
between the ONIOM layers, two linking hydrogen atoms were added between S, and Fe (of the proximal cubane), and between Cα and Cβ (of Cys382).

The UFF charge equilibration method was utilized to describe the electrostatic interactions within the MM region of DdH, whereas the solvent charges were obtained from literature132 (i.e., qO = -0.706 a.u., and qH = 0.353 a.u.).

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. Then, after each substitution, geometry optimization is performed, followed by frequency calculations to obtain the Gibbs’ energy of H2O dissociation.

The Pymol133 program has been employed to measure interatomic distances between oxygen (of the exogenous H2O) and terminal atoms (excluding hydrogens) of residue R-groups; it was also used for residue substitutions. The Swiss PDB Viewer134 was utilized to add protons to obtain protonated histidines.
Lastly, Gibbs’ energies of H₂O and H₃O⁺ (viz., ΔG₇6.419750, and ΔG₇6.598767 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.

4.4. Results

4.4.1. Thermodynamic Analysis

The reactivation mechanism of [Fe-Fe]-hydrogenase H-cluster essentially consists of three reaction steps, viz., protonation, reduction, and H₂O elimination. The reactivation pathways (Scheme 4-1, 4-2, and 4-3) proceed with different combinations for the three steps.
Scheme I for H-cluster Reactivation

Scheme 4-1. The reactivation pathway I of [Fe-Fe]-hydrogenase H-cluster.
Scheme II for H-cluster Reactivation

Scheme 4-2. The reactivation pathway II of [Fe-Fe]-hydrogenase H-cluster.
Scheme III for H-cluster Reactivation

Scheme 4-3. The reactivation pathway III of [Fe-Fe]-hydrogenase H-cluster.
For the hybrid calculations, the reductive step, 1 → 2 (Scheme 4-1), of [Fe$^{II}$-Fe$^{II}$]-hydrogenase H-cluster proceeds rather endergonically, viz., $\Delta G_{\text{Enzyme}} = +42.6$ kcal/mol (Table 4-I) relative to the gas phase ($\Delta G_{\text{Gas } \Phi} = +8.2$ kcal/mol, Table 4-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 4-1), is highly exergonic ($\Delta G_{\text{Enzyme } \Phi} = -317.9$ kcal/mol). The Gibbs’ energy difference between the reactions of the two environments, (i.e., vacuum vs. enzyme $\Phi$, Table 4-I), is +91.3 kcal/mol, and points to a more spontaneous reaction in gas phase. In step 3 → 4 (H$_2$O elimination), the hydrogenase H-cluster calculations show a rather small, endergonic result ($\Delta G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol), as opposed to the exergonic gas phase outcome ($\Delta G_{\text{Gas } \Phi} = -6.6$ kcal/mol). The enzymatic H$_2$O removal is nonspontaneous due to the influence of the protein environment on the H-cluster electronic properties.

The DdH protonation, 1 → 2' (Scheme 4-2), provides an exergonic reaction, viz., $\Delta G_{\text{Enzyme } \Phi} = -236.8$ kcal/mol, proceeding less spontaneously than in gas phase, viz. $\Delta G_{\text{Gas } \Phi} = -328.3$ kcal/mol, correlating the trend in Scheme 4-1. The enzyme $\Phi$ calculations, for the H$_2$O removal, 2' → 3', (Scheme 4-2), confer an endergonic result, ($\Delta G_{\text{Enzyme } \Phi} = +22.9$ kcal/mol), as opposed to the exergonic gas $\Phi$ reaction step ($\Delta G_{\text{Gas } \Phi} = -16.6$ 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' → 4, of the aqueous enzyme $\Phi$ (Scheme 4-2) proceeds exergonically, viz., $\Delta G_{\text{Enzyme } \Phi} = -59.4$ kcal/mol, close to the gas phase result, viz., $\Delta G_{\text{Gas } \Phi} = -62.7$ kcal/mol.
However, Scheme 4-3, 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., $\Delta G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol, which could be changed into an exergonic reaction via mutagenesis. For Scheme 4-3, only the H$_2$O removal step is endergonic (viz., $\Delta G_{\text{Enzyme } \Phi} = +2.0$ kcal/mol, as in Scheme 4-1), whereas the other remaining steps [protonation (viz., $\Delta G_{\text{Enzyme } \Phi} = -236.8$ kcal/mol), reduction (viz., $\Delta G_{\text{Enzyme } \Phi} = -38.4$ 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 4-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 4-1, the reduction step, 1 $\rightarrow$ 2, upon the removal of Glu$^{240}$, becomes less endergonic by $\Delta G = +17.0$ kcal/mol (relative to wild-type DdH) but not even close to make it spontaneous$^9$. For 2 $\rightarrow$ 3, with the removal of a basic residue, i.e., Lys$^{237}$ (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 $\rightarrow$ 4, proceeds

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$^9$To improve the electron transfer step, given that a negative residue (such as Glu$^{240}$) has been removed, one should proceed towards the positively charged residues, i.e., basic residues.
exergonically, viz., $\Delta G_{\text{Enzyme}} = -1.6$ kcal/mol, upon the removal of Glu$^{374}$ from the apoenzyme, however Scheme 4-1 cannot proceed to completion due to the endergonic, reductive step, $1 \rightarrow 2$.

For Scheme 4-2, an improvement has been obtained (as in Scheme 4-1) upon the removal of exactly the same residue for each of the corresponding step, i.e., Lys$^{237}$ for protonation (viz., $\Delta G_{\text{Enzyme}} = -264.4$ kcal/mol), Glu$^{240}$ for reduction (viz., $\Delta G_{\text{Enzyme}} = -78.7$ kcal/mol), and Glu$^{374}$ for H$_2$O elimination (viz., $\Delta G_{\text{Enzyme}} = +21.5$ kcal/mol). However, in spite of Gibbs’ energy improvements for all reaction steps, no matter what residues (Table 4-I) are removed, Scheme 4-2 is hindered from completion in the H$_2$O removal step.

In Scheme 4-3, 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 $\Delta$Arg$^{111}$, $\Delta$Thr$^{145}$, $\Delta$Ser$^{177}$, $\Delta$Glu$^{240}$, $\Delta$Glu$^{374}$, and $\Delta$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 4-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 4-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.

### 4.4.2. Geometric Considerations

In order 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 H$_2$O removal, (3 $\rightarrow$ 4), reveal a contrasting picture regarding CO$_b$ translation towards Fe$_d$ relative to the gas phase H$_2$O elimination. That is, in Table 4-III$^0$, 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 $\Phi$ distance becomes smaller (viz., 1.945 Å $\rightarrow$ 1.850 Å), and the reaction (3 $\rightarrow$ 4) is exergonic]$^2, 87$, which may explain why H$_2$O removal is endergonic for the enzyme $\Phi$.

Thus, it is ascertained that for the aqueous enzyme $\Phi$, an endergonic ligand (H$_2$O) 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 H$_2$O elimination.

$^0$In gas $\Phi$, both Scheme 4-1and 4-2 for H$_2$O 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 4-1, and 4-2, respectively. Hence, the shifting of the CO$_b$ towards Fe$_d$ seems to facilitate the exogenous ligand removal, e.g., H$_2$O, and H$_2$. 
Out of the three reaction pathways presented here, only Scheme 4-3 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 Feₚ and Feₜ, Feₚ and COᵦ, Feₜ and COᵦ, and Feₜ 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 Feₜ and COᵦ is slightly increasing (Table 4-III), which corresponds to the endergonic step, viz., ΔG<sub>Enzyme</sub>ₜ = +2.0 kcal/mol. However, the H₂O removal from the mutated enzyme is exergonic (Table 4-II), correlating to the movement of the COᵦ towards the Feₜ. As a result of mutating DdH, the following bond contractions, Feₜ-COᵦ, have been obtained, viz., 0.024 Å for Arg<sup>111</sup>Glu, 0.028 Å for Thr<sup>145</sup>Val, 0.017 Å for Glu<sup>374</sup>His, and 0.031 Å for Tyr<sup>375</sup>Phe, corresponding to exergonic steps for water elimination (Table 4-II).

Note that a simultaneous bond elongation occurs [for all present mutations (Table 4-III)] between the Feₚ and the bridging carbonyl, Feₚ-COᵦ, when bond contraction for Feₜ-COᵦ takes place. As a result of mutating DdH (vs. the wild-type enzyme), larger bond contractions between the iron atoms, Feₜ-Feₚ, have been obtained (ca. 0.1 Å, Table 4-III). It is also noticed that the bond length between the distal iron and water, Feₜ-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 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 compared to Glu$^{374}$His mutant where the substituted amino acid (at a greater distance from H$_2$O) is not only polar but of opposite charge as well.

The negative partial charge on oxygen (-0.935) of H$_2$O 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.
Figure 4-2. The frontier molecular orbitals from DFT calculations (B3LYP/6-31+G(d,p)).
4.4.3. 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 4-1, 1 → 2), and according to the LUMO depiction (Figure 4-2), the transferred e− appears to be localized in the vicinity of the di-iron atoms, Fe-Fe. However, according to the NBO partial charge results (Figure 4-3), the iron atoms (of hydrogenase H-cluster 1) do not have affinity for the approaching e−, i.e., qFe = -1.291 a.u., and qFe = -1.109 a.u.. The fact that the reduction is endergonic (ΔG_{Enzyme Φ} = +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.
Figure 4-3. 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.
### Table 4-I: Native and Residue Removed DdH Gibbs' Energies (kcal/mol) for the Reaction Steps of Reactivation Pathways I, II, and III

<table>
<thead>
<tr>
<th>Reaction steps</th>
<th>(+e)</th>
<th>(+H^+)</th>
<th>(-H_2O)</th>
<th>(+H^+)</th>
<th>(-H_2O)</th>
<th>(+e)</th>
<th>(+e)</th>
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<tbody>
<tr>
<td>Native DdH</td>
<td></td>
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<td>ΔSer^{325s}*</td>
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<td>-236.8</td>
<td>+22.9</td>
<td>-59.4</td>
<td>-38.4</td>
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<td>-251.4</td>
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<td>-44.7</td>
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<td>+1.6</td>
<td>-241.1</td>
<td>+22.8</td>
<td>-54.8</td>
<td>-33.6</td>
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<td>-305.0</td>
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<td>-223.4</td>
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<td>-52.8</td>
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<tr>
<td>ΔThr^{145}</td>
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<td>-0.2</td>
<td>-237.9</td>
<td>+22.8</td>
<td>-59.9</td>
<td>-36.9</td>
</tr>
<tr>
<td>ΔGlu^{146}</td>
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<td>-38.5</td>
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<td>+22.7</td>
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</tr>
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<td>-56.0</td>
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<td>ΔAsn^{207}</td>
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<td>+1.5</td>
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<td>-264.4</td>
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<td>ΔLys^{238}</td>
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<tr>
<td>ΔThr^{259}</td>
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<td>+1.8</td>
<td>-239.9</td>
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<td>+23.0</td>
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<td>ΔGlu^{374}</td>
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<td><strong>-1.6</strong></td>
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<td><strong>-76.5</strong></td>
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<td>ΔTyr^{375}</td>
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<td>Gas φ</td>
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*Residue removed DdH; s = small chain
Table 4-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</th>
<th>3 → 4</th>
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<tr>
<td>Arg¹¹¹Glu</td>
<td>-0.9</td>
</tr>
<tr>
<td>Thr¹⁴⁵Val</td>
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</tr>
<tr>
<td>Ser¹⁷⁷Ala</td>
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<tr>
<td>Glu²⁴⁰His</td>
<td>+1.1</td>
</tr>
<tr>
<td>Glu³⁷⁴His</td>
<td>-3.1</td>
</tr>
<tr>
<td>Tyr³⁷⁵Phe</td>
<td>-2.1</td>
</tr>
<tr>
<td>Combinations of:</td>
<td></td>
</tr>
<tr>
<td>Glu³⁷⁴His and Tyr³⁷⁵Phe</td>
<td>-5.1</td>
</tr>
<tr>
<td>Thr¹⁴⁵Val, Glu³⁷⁴His and Tyr³⁷⁵Phe</td>
<td>-6.2</td>
</tr>
<tr>
<td>Arg¹¹¹Glu, Thr¹⁴⁵Val, Glu³⁷⁴His and</td>
<td></td>
</tr>
<tr>
<td>Tyr³⁷⁵Phe</td>
<td>-7.5</td>
</tr>
</tbody>
</table>
Regarding [Fe-Fe]-hydrogenase H-cluster 2, \((2 \rightarrow 3)\), the open shells, SOMO\(_{\alpha}\), HOMO\(_{\alpha}\), and HOMO\(_{\beta}\), have similar orbital distribution over OH\(^-\) (Figure 4-2), and could make a σ-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\(_{\alpha}\), HOMO\(_{\beta}\), and SOMO\(_{\alpha}\), are diffused throughout the cluster except over the Fe\(_d\)-OH\(_2\) bond, implying that the e’s of the σ-bond, Fe\(_d\)-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, i.e., \(\Delta G = +2.0\) kcal/mol.

For [Fe-Fe]-hydrogenase H-cluster 1, (Scheme 4-2, \(1 \rightarrow 2'\)), 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 (\(q_O = -0.898; q_N = -0.682\)). Note that these analyses also agree with the calculated hydrogenase H-cluster proton affinity, i.e., \(\Delta G = -236.8\) kcal/mol.

For H-cluster 2’, (Scheme 4-2, \(2' \rightarrow 3'\)), 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\(_2\)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, i.e., \(\Delta G = +22.9\) 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 → 1.942 Å [vs. 1.907 → 1.908 Å (in H-cluster 3, Scheme 4-
1, 3 → 4)], which partially accounts for the more endergonic H₂O elimination process, coinciding with an increase of one order of magnitude for the Gibbs’ energy difference (for water elimination in 2' → 3' vs. 3 → 4).

In H-cluster 3', LUMO is mostly localized on the potential catalytic binding site. Upon the reduction of the H-cluster, (3' → 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_Fep = -1.317 a.u., and q_Fed = -0.733 a.u., in conjunction with the above presented LUMO depiction, the cluster reduction occurs with a rather high spontaneity, i.e., $\Delta G_{\text{Enzyme} \Phi} = -59.4$ 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 (Figure 4-2). Cluster reduction (Scheme 4-3, 2' → 3) occurs with a rather high spontaneity, i.e., $\Delta G_{\text{Enzyme} \Phi} = -38.4$ kcal/mol, although the di-iron atoms have high NBO negative charges, i.e., q_Fep = -1.305 a.u., and q_Fed = -1.077 a.u. [just as for H-cluster 3', (3' → 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 → 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.

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1Because 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 (Fe₄S₄/Cys₄) cluster.
Table 4-III. Interatomic distances (Å) for wild-type and mutated DdH, between Fe\textsubscript{p} and Fe\textsubscript{d}, Fe\textsubscript{p} and CO\textsubscript{b}, Fe\textsubscript{d} and CO\textsubscript{b}, and Fe\textsubscript{d} and H\textsubscript{2}O, before and after H\textsubscript{2}O removal (3 → 4)

<table>
<thead>
<tr>
<th></th>
<th>Before H\textsubscript{2}O removal</th>
<th>3 (Wild-type)</th>
<th>3 (Arg\textsuperscript{111} Glu mutant)</th>
<th>3 (Thr\textsuperscript{145} Val mutant)</th>
<th>3 (Glu\textsuperscript{374} His mutant)</th>
<th>3 (Tyr\textsuperscript{375} Phe mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe\textsubscript{p}-Fe\textsubscript{d}</td>
<td>2.626</td>
<td>2.659</td>
<td>2.670</td>
<td>2.690</td>
<td>2.666</td>
<td></td>
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<tr>
<td>CO\textsubscript{b}-Fe\textsubscript{p}</td>
<td>1.959</td>
<td>1.963</td>
<td>1.968</td>
<td>1.975</td>
<td>1.991</td>
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<tr>
<td>CO\textsubscript{b}-Fe\textsubscript{d}</td>
<td>1.907</td>
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<td>1.963</td>
<td>1.945</td>
<td>1.937</td>
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<tr>
<td>Fe\textsubscript{d}-O(H\textsubscript{2}O)</td>
<td>2.127</td>
<td>2.205</td>
<td>2.181</td>
<td>2.223</td>
<td>2.184</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>After H\textsubscript{2}O removal</th>
<th>4 (Wild-type)</th>
<th>4 (Arg\textsuperscript{111} Glu mutant)</th>
<th>4 (Thr\textsuperscript{145} Val mutant)</th>
<th>4 (Glu\textsuperscript{374} His mutant)</th>
<th>4 (Tyr\textsuperscript{375} Phe mutant)</th>
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<tbody>
<tr>
<td>Fe\textsubscript{p}-Fe\textsubscript{d}</td>
<td>2.587</td>
<td>2.584</td>
<td>2.616</td>
<td>2.589</td>
<td>2.582</td>
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<td>CO\textsubscript{b}-Fe\textsubscript{p}</td>
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<td>CO\textsubscript{b}-Fe\textsubscript{d}</td>
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<td>1.935</td>
<td>1.928</td>
<td>1.906</td>
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Table 4-IV. Interatomic distances between the oxygen (of exogenous H$_2$O; Fe$_0$-OH$_2$, compound 3) and the juxtaposed atoms of the residue R group

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<tr>
<th>Mutated DdH</th>
<th>H$_2$O oxygen</th>
<th>Å</th>
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<tbody>
<tr>
<td>Arg$^{111}$ Glu mutant</td>
<td>O(ε)</td>
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<td>O(ε)</td>
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<tr>
<td>Thr$^{145}$ Val mutant</td>
<td>C(γ)</td>
<td>9.616</td>
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<tr>
<td></td>
<td>C(γ)</td>
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<tr>
<td>Ser$^{177}$ Ala mutant</td>
<td>C(β)</td>
<td>6.077</td>
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<tr>
<td>Glu$^{240}$ His mutant</td>
<td>N(δ)</td>
<td>12.395</td>
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<tr>
<td></td>
<td>N(ε)</td>
<td>11.666</td>
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<tr>
<td>Glu$^{374}$ His mutant</td>
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<td></td>
<td>N(ε)</td>
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<tr>
<td>Tyr$^{375}$ Phe mutant</td>
<td>C(ζ)</td>
<td>7.724</td>
</tr>
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</table>
4.5. Discussion

The comparison between gas phase and wild-type enzyme calculations, presented in the current study, unambiguously shows that CO\textsubscript{b} migration is key to enzyme inhibition by O\textsubscript{2}. When CO\textsubscript{b} is close to the Fe\textsubscript{d}, DdH becomes reactivated, while, on the other hand, when CO\textsubscript{b} is further away from Fe\textsubscript{d}, wild-type enzyme H-cluster inactivation is observed. The displacement of CO\textsubscript{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\textsubscript{b} away from the catalytic site, Fe\textsubscript{d}, leading to an exogenously inhibited hydrogenase, and thus hindering H\textsubscript{2}O elimination. On the contrary, suitable residue substitutions can reverse the enzyme inhibition by allowing CO\textsubscript{b} to migrate towards Fe\textsubscript{d}, concomitant with H\textsubscript{2}O removal.

The potential reactivation pathway of Scheme 4-1 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 4-2) cannot be realized because of the high nonspontaneous step for H\textsubscript{2}O removal (2' → 3', \(\Delta G_{\text{Enzyme}} = +22.9\) kcal/mol). As a result, this scheme does not seem to provide room for mutagenic enhancements (residue substitutions) that could improve H\textsubscript{2}O 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 of pathway III provides the best chance for reactivating DdH, because the only reaction step to overcome (H₂O removal) is barely endergonic (ΔG_{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., Arg^{111} Glu, Thr^{145} Val, Glu^{374} His, and Tyr^{375} Phe providing ΔG = -7.5 kcal/mol).

4.6. Conclusions

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

For reactivation pathway II, the [Fe-Fe]-hydrogenase H-cluster H⁺ transfer occurs first (ΔG_{Enzyme \Phi} = -236.8 \text{ kcal/mol}), followed by H₂O removal (ΔG_{Enzyme \Phi} = +22.9 \text{ kcal/mol}), and then by e⁻ transfer (ΔG_{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., ΔG_{Enzyme \Phi} = -236.8 \text{ kcal/mol}), an exergonic e⁻ transfer step (viz., ΔG_{Enzyme \Phi} = -38.4 \text{ kcal/mol}), followed by water removal step (viz., ΔG_{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 H$_2$O removal, was made to proceed more spontaneously by removing inhibitory residues, one at a time. The promising residues are ΔArg$^{111}$, ΔThr$^{145}$, ΔSer$^{177}$, ΔGlu$^{240}$, ΔGlu$^{374}$, and ΔTyr$^{375}$, 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., Arg$^{111}$Glu, Thr$^{145}$Val, Ser$^{177}$Ala, Glu$^{240}$His, Glu$^{374}$His, and Tyr$^{375}$Phe. All substitutions resulted in improved spontaneity for H$_2$O removal, relative to residue deletions, except for ΔSer$^{177}$ → Ser$^{177}$Ala (ΔG = -0.5 kcal/mol → ΔG = -0.1 kcal/mol), and ΔGlu$^{240}$ → Glu$^{240}$His (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, Glu$^{374}$His, and Tyr$^{375}$Phe, resulted in ΔG = -5.1 kcal/mol; the three residue combination, Thr$^{145}$Val, Glu$^{374}$His, and Tyr$^{375}$Phe, provided a ΔG = -6.2 kcal/mol, and the four residue combination, Arg$^{111}$Glu, Thr$^{145}$Val, Glu$^{374}$His, and Tyr$^{375}$Phe, gave a ΔG = -7.5 kcal/mol. Though the H$_2$O 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 H$_2$O 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 H$_2$O removal; this, then, may partly explain why the removal of H$_2$O is exergonic for gas Φ, as opposed to the enzyme Φ.

Since pathway III has only the H$_2$O 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 H$_2$O removal from the wild-type hydrogenase ($\Delta G_{\text{Enzyme }\Phi} = +2.0 \text{ kcal/mol}$) shows that the distance between the Fe$_d$ 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$_d$. The following bond contractions, Fe$_d$-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, viz., ΔGlu$^{374}$ → Glu$^{374}$His ($\Delta G = -1.6 \text{ kcal/mol} \rightarrow \Delta G = -3.1 \text{ kcal/mol}$), can reactivate the [Fe-Fe]-hydrogenase.
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127. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.;
    Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.;
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    Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.;


