Enzyme Models

Aerobic Damage to [FeFe]-Hydrogenases: Activation Barriers for the Chemical Attachment of O₂

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Abstract: [FeFe]-hydrogenases are the best natural hydrogen-producing enzymes but their biotechnological exploitation is hampered by their extreme oxygen sensitivity. The free energy profile for the chemical attachment of O₂ to the enzyme active site was investigated by using a range-separated density functional re-parametrized to reproduce high-level ab initio data. An activation free-energy barrier of 13 kcal mol⁻¹ was obtained for chemical bond formation between the di-iron active site and O₂, a value in good agreement with experimental inactivation rates. The oxygen binding can be viewed as an inner-sphere electron-transfer process that is strongly influenced by Coulombic interactions with the proximal cubane cluster and the protein environment. The implications of these results for future mutation studies with the aim of increasing the oxygen tolerance of this enzyme are discussed.

Hydrogenases are enzymes that catalyze hydrogen oxidation and reduction according to the reaction H₂ + 2H⁺ + 2e⁻ → H₂O. [1] Among the various members of this enzyme family, [FeFe]-hydrogenases show the highest activity for hydrogen production, with a turnover rate constant of over 10,000 s⁻¹. [2] Although synthetic catalysts with even higher turnover rates have recently been reported, [3] hydrogenases are still un-rivalled in terms of reversibility and the low overpotential required. They have been successfully used as catalysts in biofuel cells in the laboratory, [4] but applications at an industrial scale are hampered in part by the irreversible inhibition and damage of [FeFe]-hydrogenases by molecular oxygen. [5] Some membrane-bound [NiFe]-hydrogenases [6] can operate in the presence of oxygen but they are biased towards hydrogen oxidation. [7] Hence, there are currently intense efforts ongoing to make [FeFe]-hydrogenase, the best H₂ producers, less sensitive to O₂. [8,9]

Early electrochemical studies have indicated that oxygen inhibition of [FeFe]-hydrogenases proceeds through the reversible formation of an oxygen adduct and is followed by slower irreversible damage to the enzyme. [9] Later, evidence was reported that the destruction of the enzyme is initiated by O₂ binding to one of the Fe atoms of the di-iron cluster, where it is converted to a reactive oxygen species (ROS) that subsequently destroys the neighbouring [Fe₅S₆] cubane cluster (Figure 1). [10] This picture is consistent with the theoretical work of Reiher et al. [11] who performed extensive density functional theory (DFT) studies on the regioselectivity of oxygen binding to [FeFe]-hydrogenases and on possible reaction intermediates.

In addition to the thermodynamics of O₂ binding studied in the above-mentioned works, it is crucial to understand the kinetics of this process. Overall O₂ and CO binding rates (also termed inactivation rates kₜ) have recently been measured. [12] However, it remains unclear whether these rates are limited by actual chemical bond formation between O₂ and the di-iron site or by diffusion of O₂ from the solvent to the active-site pocket. [13] Another alternative is that both steps occur on the same time scale as was indeed found for CO₂ binding to carbon monoxide dehydrogenase/acetyl-CoA synthase. [14] This knowledge is important because any attempt to reduce oxygen binding would be most effective if the slowest step is targeted.

Hence, a first step and the focus of the current work is to obtain reliable estimates for the activation barrier for the chemical attachment of O₂ to the di-iron site. This is challenging because of the complicated electronic structure of the hydrogenase active site. Herein, we address this problem through re-parametrization of a range-separated hybrid density functional theory correlated ab initio reference data at the n-electron valence state perturbation theory (NEVPT2) [15] level. The estimated activation free energy, 13 kcal mol⁻¹ for [FeFe]-hydrogenases from Clostridium pasteurianum (Cp) [16] and Desulfovibrio desulfuricans (Dd), [17] suggests that chemical bond formation constitutes a major barrier for the O₂ binding process. The calculations reveal that spontaneous electron transfer (ET) from the di-iron site to O₂ makes the oxygen attachment thermodynamically favorable.

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thus suggesting that mutations that counteract this ET may help to increase oxygen resistance.

We have investigated a large computational model for the enzyme active site (Figure 1a). It contains the di-iron site (red) and the adjacent [Fe4S4] cluster (blue), collectively referred to as the H-cluster, as well as all close-lying residues that form hydrogen bonds with atoms of the H-cluster. The models for the *Dd* (PDB id: 3C8Y) and *Cp* (PDB id: 1HFE) enzymes are almost the same except that Gly 302 and Ala 377 are included only for the *Dd* protein because they do not form hydrogen bonds in the *Cp* structure. Calculations were carried out for the catalytically active H ox state, which has been shown to be the relevant state for oxygen inhibition[10] (Figure 1b, see the Experimental Section for further details).

Geometry optimization of this large model with the BP86 functional gives very good agreement with the crystal structure (Table S3 in the Supporting Information) in line with the good performance reported previously.[18] However, it can be expected that the electron delocalization error of this density functional[19] significantly affects the activation barrier for oxygen binding. We investigated this issue in more detail and constructed an active-site model (indicated in red in Figure 1, CYS 503/382 was replaced with CH3SH) that is small enough to permit reference calculations at the NEVPT2 level of theory. The O2 binding curves obtained are shown in Figure 2. An energy barrier of 5.6 kcalmol⁻¹ and a binding energy of −7.6 kcalmol⁻¹ were obtained at the NEVPT2 level. With the BP86 functional, the potential energy curve is purely attractive and the binding energy is overestimated by 2.5 kcalmol⁻¹. Global hybrid functionals such as B3LYP predict a very small barrier at the cost of significantly reduced binding energy. This observation prompted us to parametrize a range-separated hybrid functional based on CAM-B3LYP[20] with modified parameters $\alpha = 0.1$, $\beta = 0.9$, $\mu = 0.5$ (10% Hartree Fock exchange at $r_{12} < 0$ and 100% at $r_{12} = \infty$; $r_{12}$ is the interelectronic distance) to yield a best fit to the NEVPT2 data. The corresponding functional is denoted CA1-B3LYP. It captures both the activation barrier (6.4 kcalmol⁻¹) and the binding energy (−5.1 kcalmol⁻¹) to good accuracy (Figure 2), as well as other properties such as weak interactions and hydrogen bonding (see Table S1 in the Supporting Information).

An explanation for why our range-separated functional works can be found in Figure 2b. At long distance, both the binding and anti-binding combinations of 3d½ (Fe_contrib) and σ (O2) orbitals represented by natural molecular orbitals (NMOs) 109 and 111, respectively, have occupation numbers of one. This picture is obtained with the reference calculations and with our new functional but not with BP86. In the latter case, spurious charge-transfer can be seen at all distances. The CA1-B3LYP functional follows the reference curve up to the distance of 2.3 Å, where a switch from one potential energy surface (Fe_contrib−O2) to another occurs (Fe_contrib−O2*), that can be interpreted as electron transfer from Fe_contrib to O2. Thus, the variable amount of exact exchange in the “single-reference” density functional is able to mimic the two important charge transfer configurations.

The CA1-B3LYP functional was subsequently used to calculate the activation energy ($\Delta E^*$) and binding energy

Figure 1. The active site of the [FeFe]-hydrogenases. The H-cluster is shown in blue and red, along with interacting triplet oxygen. a) the protein binding pocket of the H-cluster with all of the residues included in the large model. Amino acid abbreviations and numbers are given for *Cp* and *Dd* in regular and italic font, respectively. In the ONIOM approach, the high level was chosen as all atoms inside the closed figure. b) The spin-coupling scheme adopted in this study.

Figure 2. a) The relative energy change upon oxygen binding to the distal iron atom of the small cluster calculated by various methods. b) The change in the occupation number of the natural molecular orbitals corresponding to σ and σ* bonds between the distal iron and the oxygen molecule. Details can be found in the Supporting Information.
Further calculations on additional active-site models suggest that the presence of the highly negatively charged [Fe₄S₄] cubane cluster plays an important role in oxygen binding (see the Supporting Information). This cluster stabilizes the di-iron site, which becomes partially oxidized through donation of electron density upon O₂ attachment. The cubane cluster thus facilitates ET from Fe₃ to O₂, most probably through purely electrostatic interactions. The small difference we observed between the Cp and Dd protein models, which differed only in the number of hydrogen bonds donated to the cubane cluster, further supports this hypothesis. Therefore, the mutation of residues in the neighborhood of the cubane cluster into positively charged amino acids such as lysine should stabilize the negative charge on the cubane and make O₂ binding less favorable. Of course, this perturbation should not be too large so that the impact on catalytic activity remains reasonably small. On this basis we suggest that possible target sites for future mutation studies could include Ser357 or Thr356 (Ala236 or Ile1235 for Dd), all of which are nonconserved and in close proximity to the cubane cluster.

**Experimental Section**

The lowest-energy spin coupling defined in Figure 1b was chosen consistently with previous calculations[11,23]. Although the location of the unpaired electron (on either Fe₃ or Fe₄) is the subject of ongoing discussions, all studies agree that both iron atoms are low-spin +1 or +2[24]. We adopted the structure with a bridging CO molecule (Fe₅Fe₆ redox state).[25] In the DFT calculations, the broken-symmetry (BS) approach[26] was employed. The high negative charge of the H-cluster (−3) was compensated by three protonated proximal lysyl residues to give a total charge of zero. We considered only the antiferromagnetic coupling with triplet oxygen (Sₚ = 1/2) because at long distance, this state is essentially degenerate with the S = 3/2 state.[26] Geometry optimizations were carried out with the BP86 + D3 functional[27] and the def2-TZVP basis[28] as implemented in the Turbomole program.[29] CA1-B3LYP calculations on the BP86 + D3 structures were carried out using the ONIOM approach[30] as implemented in the NWChem program.[31] The energy of the low-level layer (entire system) was evaluated with the BP86 + D3 functional while the CA1-B3LYP functional was used for the high-level part encircled in Figure 1a. NEVPT2 calculations were carried out using the ORCA 2.9.1 package.[32] Further details and Cartesian coordinates of all relevant structures can be found in the Supporting Information.

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**References**


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