Molecular dynamics and experimental investigation of H_2 and O_2 diffusion in [Fe]-hydrogenase

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Abstract

The [Fe]-hydrogenase enzymes are highly efficient H_2 catalysts found in ecologically and phylogenetically diverse microorganisms, including the photosynthetic green alga, *Chlamydomonas reinhardtii*. Although these enzymes can occur in several forms, H_2 catalysis takes place at a unique [FeS] prosthetic group or H-cluster, located at the active site. Significant to the function of hydrogenases is how the surrounding protein structure facilitates substrate-product transfer, and protects the active site H-cluster from inactivation. To elucidate the role of protein structure in O_2 inactivation of [Fe]-hydrogenases, experimental and theoretical investigations have been performed. Molecular dynamics was used to comparatively investigate O_2 and H_2 diffusion in CpI ([Fe]-hydrogenase I from *Clostridium pasteurianum*). Our preliminary results suggest that H_2 diffuses more easily and freely than O_2, which is restricted to a small number of allowed pathways to and from the active site. These O_2 pathways are located in the conserved active site domain, shown experimentally to have an essential role in active site protection.

Introduction

Hydrogen production in the green alga, *Chlamydomonas reinhardtii*, is a strictly anaerobic process. Coupling with photosynthesis can be achieved under conditions of sulphur deprivation that results in a series of physiological events leading to reduced PSII activity, the respiratory consumption of residual O_2 and establishment of an anaerobic state [1,2]. Induction of hydrogenase gene expression and enzyme synthesis ultimately results in H_2 production. Although metabolic sequestration of O_2 overcomes the severe sensitivity of algal [Fe]-hydrogenase to O_2, the H_2 production efficiency is lower than the theoretical maximum [1,2]. To develop an efficient large-scale, photobiological H_2 production system, the O_2 sensitivity of algal [Fe]-hydrogenases needs to be addressed [1].

Inactivation of [Fe]-hydrogenase by O_2 involves diffusion of the diatomic gas from solvent to the active site, and subsequent chemical oxidation of the catalytic H-cluster [3]. The initial stages of diffusion involve molecular interactions between O_2 and protein, suggesting that the protein structure influences how O_2 approaches the active site. Previous studies of H_2 diffusion in [NiFe]-hydrogenase suggest that H_2 diffusion in the enzyme is not a random process, but rather tends to occur through a specific, pre-defined pathway, the H_2-channel [4]. To investigate further the gas diffusion in hydrogenase and to elucidate the initial events in the O_2-inactivation process, molecular mechanics methods were used to study O_2 and H_2 diffusion in CpI ([Fe]-hydrogenase I from *Clostridium pasteurianum*).

Key words: *Chlamydomonas reinhardtii*, gas diffusion, hydrogenase, molecular dynamics, oxygen sensitivity, substrate.

Abbreviations used: CpI, [Fe]-hydrogenase I from *Clostridium pasteurianum*; hH$_2$, heavy dihydrogen; HydA, *Clostridium acetobutylicum* [Fe]-hydrogenase I; LES, locally enhanced sampling.

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Methods and results

Expression and O_2-sensitivity determination of [Fe]-hydrogenase

The expression of active [Fe]-hydrogenases HydA1, HydA (*Clostridium acetobutylicum* [Fe]-hydrogenase I) and HydAΔN were performed in *Escherichia coli* as previously reported [5]. Whole cell samples were solubilized, exposed to air, and aliquots assayed for residual H_2 production activity using the reduced Methyl Viologen assay [5].

Early biochemical studies of O_2-inactivation of [Fe]-hydrogenase suggested that differences exist in the O_2 sensitivities of enzymes isolated from different sources. The partially purified [Fe]-hydrogenase from *C. reinhardtii* exhibited an IC_{50} value of approx. 1 s when exposed to atmospheric levels of O_2 [6], whereas under similar conditions purified CpI exhibited an IC_{50} of 120–300 s [7] (Table 1). Our recombinantly expressed *C. reinhardtii* HydA1 [Fe]-hydrogenase was approx. 415-fold more sensitive to atmospheric O_2 (21%) than the *Clostridium acetobutylicum* HydA (Table 1). To date the algal enzymes represent the simplest [Fe]-hydrogenases known, consisting solely of an active site domain. In contrast, the homologous bacterial enzymes, CpI and HydA, are more complex, possessing an

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Table 1 | **Comparison of algal and bacterial [Fe]-hydrogenase O_2 sensitivities**

[Fe]-hydrogenase	IC_{50} value (s)
C. reinhardtii hydrogenase	<1*
C. reinhardtii HydA1	<1
Clostridium pasteurianum CpI	120–300†
Clostridium acetobutylicum HydA	415 ± 115
Clostridium acetobutylicum HydAΔN	145 ± 45

*From [6].
†From [7].

additional electron-transfer domain [8]. This domain may also contribute to protection of the active site, and may explain the higher observed O_2-tolerance levels of these enzymes. Removal of the electron-transfer domain from HydA (HydAΔN), however, resulted in only a 3-fold decrease in O_2 tolerance (Table 1), and remained approx. 140-fold more tolerant to O_2 than HydA1. The differences in enzyme sensitivities conferred by the conserved active site domain strongly suggests that the amino acid composition of this domain is critical to protection of the H-cluster from O_2.

Simulation setup and methods

Our CpI model is based on the X-ray crystal structure of CpI [8]. Missing H-cluster atoms from the CpI structure are modelled as a di(thiomethyl)amine as in [9]. The partial charges for the rest of H-cluster atoms were based on [10], with modifications of up to $\pm 0.02e$ to preserve charge neutrality. The model was then embedded in a water box, resulting in a 57 000-atom system consisting of 9000 hydrogenase atoms, 16 000 water molecules and 15 sodium ions. The full system was then equilibrated at constant temperature (310 K) and pressure (1 atm) for 1 ns.

Oxygen and hydrogen gas diffusion in CpI were investigated by all-atom molecular dynamics simulations of the outward diffusion of either O_2 or H_2 from the active site, in a similar manner to previous studies of gas diffusion across proteins [4,11]. A charge-free model for O_2 and H_2 is used. In addition, for reasons of numerical stability, and to facilitate comparison between the two gases, H_2 with equivalent mass to O_2, which we call heavy di-hydrogen (hH_2), was used in place of H_2. This way, O_2 and H_2 differ solely by their van der Waals (Lennard–Jones) parameters, molecular bond lengths and spring constants.

To increase the sampling, the method of LES (locally enhanced sampling), otherwise known as the time-dependent Hartree approximation [12], was used to simulate 1000 mutually invisible, simultaneous copies of the gas molecules (hH_2 or O_2) within a single protein-water system. Gas temperatures were regulated independently at 310 K. Each of the 1000 copies of either O_2 or hH_2 was initially placed at the same location as the H-cluster-bound CO molecule in the CpI structure [4]. For hH_2, our simulations correspond to the realistic transport of H_2 out of the protein, but for O_2, we are observing the reverse of the normal O_2 diffusion from the bulk solvent to the active site.

LES simulations were performed with NAMD2 [13] using the CHARMM22 force-field [14,15]. A constant volume and temperature were used. The system was simulated with periodic boundary conditions using Particle–Mesh Ewald method.

Molecular dynamics simulation of H_2 and O_2 diffusion in CpI

During the 1 ns equilibration, we observed a permanent and almost continuous tunnel-shaped cavity (using a 1 Å-radius probe, 1 Å = 0.1 nm) connecting the active site binding location to the solvent outside the protein at the location of the 'H_2-channel' detailed for the *Desulfovibrio desulfuricans* [Fe]-hydrogenase reported by Nicolet et al. [9,16].

Our preliminary simulations (six or four independent 2 ns simulations, for either O_2 or H_2 diffusion respectively) suggest that the diffusion pathways of O_2 and hH_2 between the active site of CpI and the protein-solvent interface are different. More specifically, on one hand, O_2 is observed to transit across the protein barrier through a limited set of precisely defined channels, of which one is displayed in Figure 1(a). We have observed O_2 motion across only two

Figure 1 | **Sample trajectory of molecular diffusion of 1000 simultaneous and mutually invisible copies of di-oxygen (O_2) from the active site of CpI hydrogenase**

Shown is (**a**) a superposition of all the O_2 positions over the 2.3 ns trajectory and snapshots of the O_2 trajectories after (**b**) 20 ps and (**c**) 2.3 ns. Figures were made using VMD (visual molecular dynamics) [17].

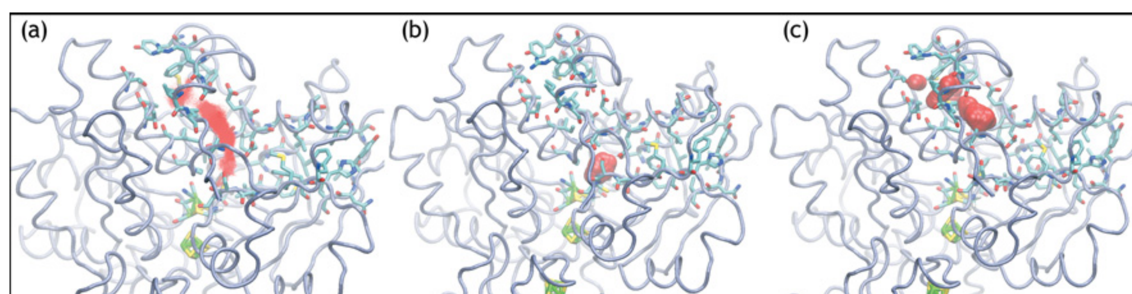

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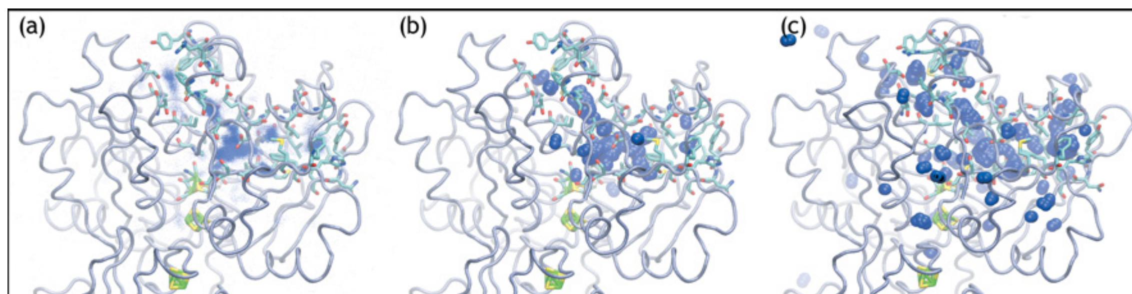
Figure 2 | Same as in Figure 1 for hH_2 diffusion

Shown is (**a**) a superposition of all the hH_2 positions of the 2.3 ns trajectory and snapshots of the hH_2 trajectories after (**b**) 300 ps and (**c**) 2.2 ns.



such channels, and in only one simulation did O_2 reach the solvent within the simulated time of 2 ns. On the other hand, in all four H_2 simulations, H_2 was observed exiting the protein. A sample of the allowed hH_2 pathways is shown in Figure 2(a). In addition, hH_2 is occasionally observed (in 2–15% of all observed exits per simulation) to cut through the bulk protein by unique paths where no channel has been detected. A second important difference between H_2 and O_2 diffusion is that, during LES simulations with 1000 copies of the gas molecule sharing a unique protein trajectory, the different simultaneous hH_2 molecules are observed to spread out in the protein and take a multitude of exits (see Figures 2b and 2c). Oxygen molecules, in stark contrast, stay clumped together for most or all of the duration of the trajectories (see Figures 1b and 1c).

Conclusion

In summary, we have shown that the diffusion of H_2 and O_2 gases inside of a [Fe]-hydrogenase is governed by the physical properties of both the gas itself and the protein structure. The dynamic process of gas migration dictates that static representations of structures can unsatisfactorily predict pathway selection. In the present study, although a majority of H_2 was found to migrate through a series of conserved hydrophobic cavities, or a 'H_2-channel' [16], H_2 is clearly able to diffuse during the same time period through a number of alternative routes. This is somewhat inconsistent with previous results shown for H_2 diffusion in [NiFe]-hydrogenase [4].

In contrast with H_2 diffusion, which displays a degree of randomized behaviour, O_2 diffusion is clearly limited to specific regions located within the conserved active site domain. The importance of this domain in O_2 diffusion agrees with the experimental results obtained in the present study. The range of sensitivity levels found for algal and bacterial enzymes are also characterized by diversity among the amino acids that comprise the diffusion pathways. From the standpoint of engineering O_2 tolerance in [Fe]-hydrogenases, the sequences and structures of naturally occurring enzymes offer important clues on how to overcome the O_2 sensitivity of algal [Fe]-hydrogenases.

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