Molecular Dynamics Investigation of O_2 Diffusion Mechanisms in the Protective *CbA5H* Hydrogenase

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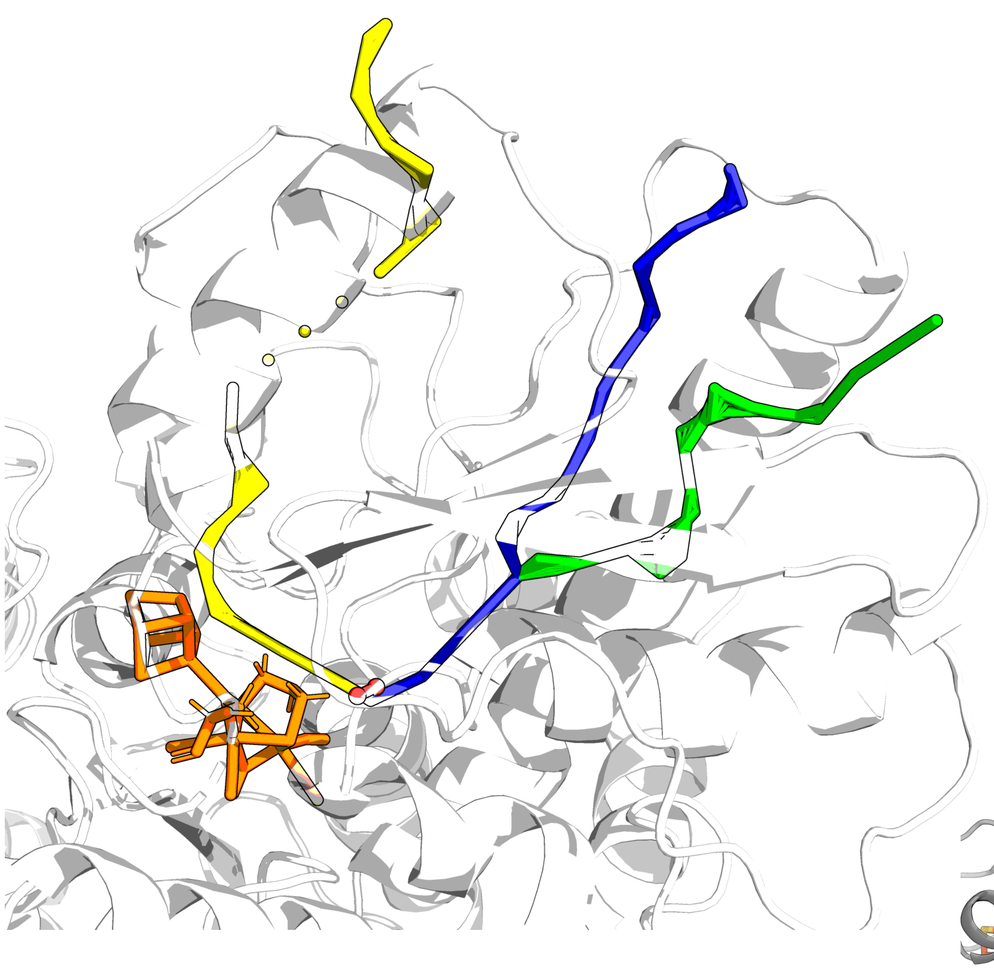
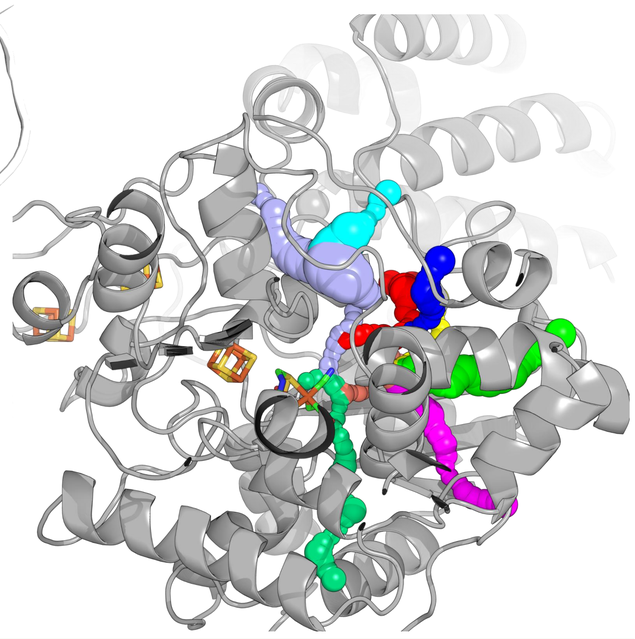
ABSTRACT

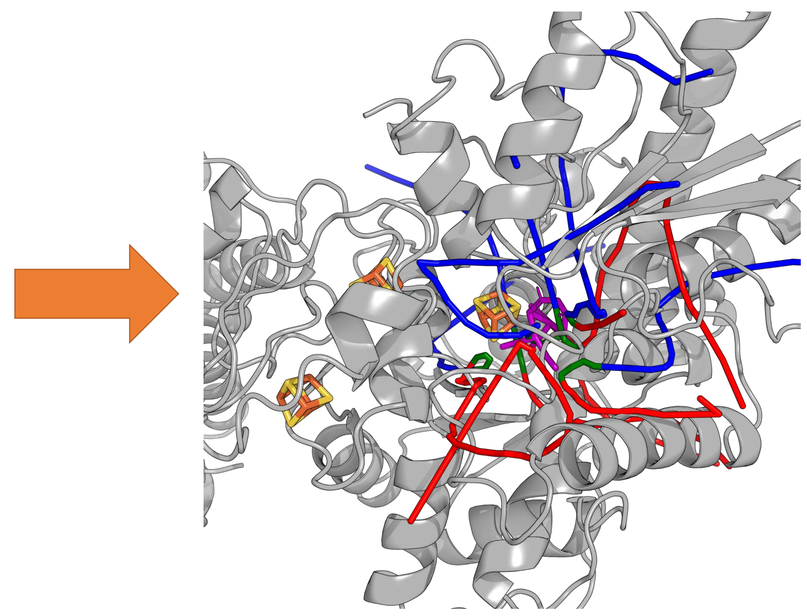
[FeFe]-hydrogenases are highly efficient biocatalysts for H_2 conversion but are extremely sensitive to O_2. The *Clostridium beijerinckii* [FeFe]-hydrogenase (*CbA5H*) displays a unique O_2-protective mechanism, forming an inactive yet rapidly reversible state upon O_2 exposure. Experimental evidence indicates that the TSC-loop enables Cys367 to complete the electronic configuration of the distal iron of the H-cluster. However, the pathways by which O_2 reaches the active site remain unclear. Here, we investigate O_2 diffusion using advanced molecular dynamics simulations (RAMD) to identify possible intraprotein diffusion routes. Our results provide molecular insight into the O_2-protection mechanism of *CbA5H* and may support protein engineering efforts toward O_2-tolerant [FeFe]-hydrogenases for sustainable H_2 production.



SIMULATION SETUP

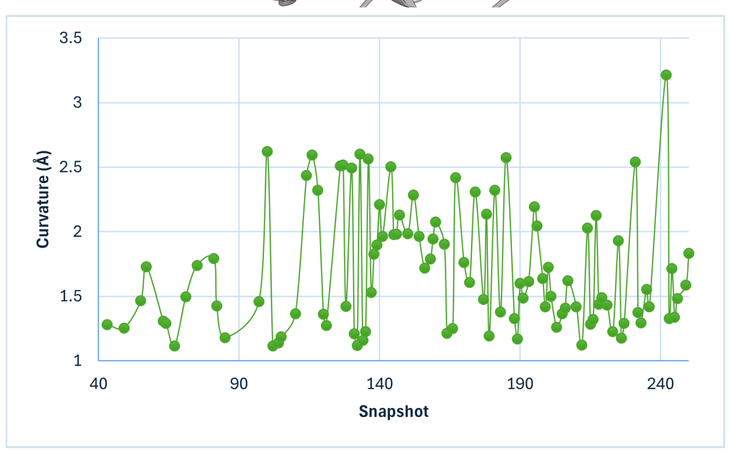
- All molecular dynamics simulations were performed using GROMACS 2024.2 and the CHARMM36 force field.
- The starting structure corresponded to the H_{ox} state of CbA5H (PDB ID: 8ZQD[1]). To investigate oxygen diffusion, a second system was generated by randomly replacing water molecules with 100 O_2 molecules.
- For each system (WT and WT-O_2), two independent molecular dynamics simulations were carried out in the NpT ensemble.
- Random Acceleration Molecular Dynamics (RAMD)[2] simulations were performed starting from the configuration containing the O_2 molecule closest to the distal iron atom (Fe_d) of the H-cluster.

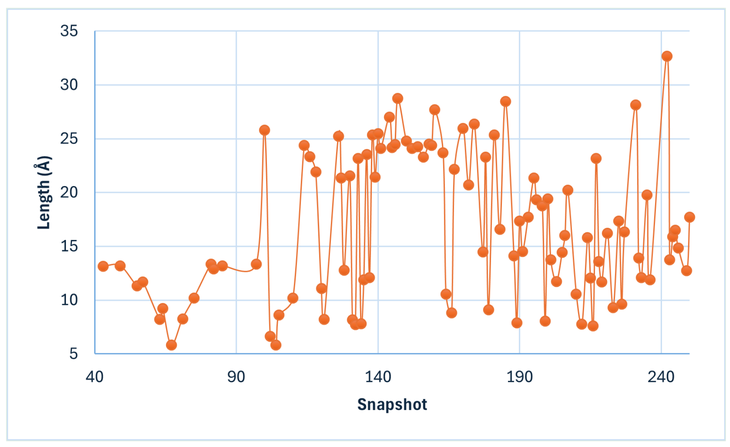




RESULTS

- RAMD simulations reveal multiple pathways for O_2
- Analysis on unbiased MD simulations reveal more stability for WT-O_2 system than WT system
- Aqua-Duct analysis reveals multiple trajectories converging toward the H-cluster region, with several paths passing in close proximity to the active site
- Structural analysis of *cluster_1* shows negligible variations in tunnel length and curvature along the simulation, indicating high geometrical stability





ID	N°_snaps	Avg_BR [Å]	SD	Max_BR [Å]	Avg_L [Å]	SD	Avg_C	SD	Priority	Avg throughput	SD
Cluster_1	102	0.885	0.146	1.17	16,762	6,657	1.7	0.468	0.17134	0.42163	0.15653
Cluster_2	80	0.724	0.08	1.12	24,506	6,917	1.81	0.371	0.0667	0.20927	0.09455
Cluster_3	71	0.859	0.154	1.21	16,218	8,757	1,417	0.582	0.12061	0.42637	0.14691

Legend: Avg_BR [Å]: Average bottleneck radius; Max_BR[Å]: maximum bottleneck radius; Avg_L [Å]: Average length; Avg_C: Average Curvature.

TAKE HOME MESSAGES

- Our simulations identify a limited number of stable and recurrent protein tunnels in *CbA5H*
- *Cluster_1* emerging as a dominant diffusion pathway toward the H-cluster. The persistence and structural stability of this tunnel support its functional relevance in O_2 migration and provide molecular insight into the intrinsic O_2-protection mechanism of *CbA5H*.
- Future work will employ advanced molecular dynamics techniques to quantitatively characterize O_2 diffusion kinetics and free-energy profiles, as well as to assess the impact of key residues, including Cys367, on tunnel accessibility and active-site protection. These approaches may guide rational engineering strategies toward O_2-tolerant [FeFe]-hydrogenases.

References

[1] Duan, J., *et al.* «Structural determinants of oxygen resistance and Zn^{2+}-mediated stability of the [FeFe]-hydrogenase from *C. beijerinckii*, *Proc. Natl. Acad. Sci. U.S.A.* 122 (3) e2416233122»

[2] *Ding, Y., et al.* «A theoretical framework for random acceleration molecular dynamics simulations». *J. Chem. Phys.* 14 February 2026; 164 (6): 064109