Project Title:

Investigating the Hydration of the H-Pathway in Bovine Heart Cytochrome *c* Oxidase

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1. Background and purpose of the project, relationship of the project with other projects

Cytochrome *c* Oxidase (C*c*O) [1], also known as Complex IV, is a key membrane protein in the respiratory process. Located in the inner mitochondrial membrane (IMM) it uses energy rich electrons from the citric acid cycle, which through the other proteins in the respiratory transport chain are transferred to C*c*O, to reduce oxygen. The energy harvested from the reduction of oxygen is used to pump protons across the IMM, thereby creating a pH gradient across the membrane important for other processes in the mitochondria.

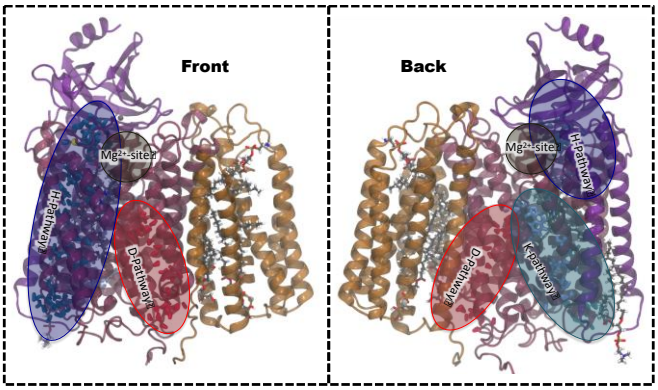


Figure 1: The A,B, C subunits of the reduced form of BHC*c*O (PDBID: 5B1B). The locations of the three proton pathways, K, D, and H, are marked on the figure. The Mg^{2+} site is believed to be the proton loading site, where the proton rests before being transferred across the IMM.

C*c*O from different organisms contain a different number of pathways, and two of these have been extensively studied, both experimentally and by simulation, namely the K, found in all C*c*O's, and the D, Found in C*c*O's belonging to the A and B families, pathways. See figure 1 for the location of these pathways in Bovine Heart C*c*O (BHC*c*O). The pathways are located in similar geometric positions in other C*c*Os, however the number of pathways and the residues lining the pathways might be different. For a more detailed discussion of the reaction mechanism and pathways of C*c*Os, see the recent review by S. Yoshikawa and A. Shimada [1].

The solution of the crystal structure of BHC*c*O revealed water molecules in regions of the protein not related to the D and K pathways [2]. It was therefore suggested that these water molecules were path of a unique pathway, which was named the H pathway, based on His413 which is believed to be located in the entry of the proton pathway.

The presence of a H pathway has been questioned by experimental studies [3] and recently also by molecular dynamics (MD) simulations [4]. Experimental studies have proven inconclusive, with some mutation studies showing that proton pumping is abandoned by single residue mutations in both the D and H pathways. The main argument against the H pathway presented in the simulation studies are that no water is found in the lower parts of the pathway under a large set of redox and protonation conditions for the protein, thereby making proton transport via the Grotthuss mechanism impossible.

These conclusions are based on the result of regular MD simulations, and can therefore be prone to the issue of insufficient sampling of relevant conformations, and by extension water populations of the H pathway. In this study we aim to find the free energy barriers of additional hydration of the lower parts of the H pathway through constant force pulling simulations. These simulations will target the interactions between the pathway walls and water molecules directly, thereby providing an

efficient sampling of the hydration of the pathway directly.

The regular MD simulations done in this project are a continuation of the simulations done in project number G17037, and the progress reached in this project would therefore not have been possible without the data generated using the resources from the previous project.

2. Specific usage status of the system and calculation method

At the time of writing all allocated resources on GWMPC (3,027,456 core hours) has been used, and around 90 % of the allocated resources on BWMPC (2,943,360 core hours) has been used.

For all calculations the locally developed Genesis program [5] were used. This project further required that a method for running constant force pulling simulations to be implemented in the Genesis program. To validate this method some of the resources on BWMPC was used for testing the method on water molecules being pulled through the water channels of aquaporin.

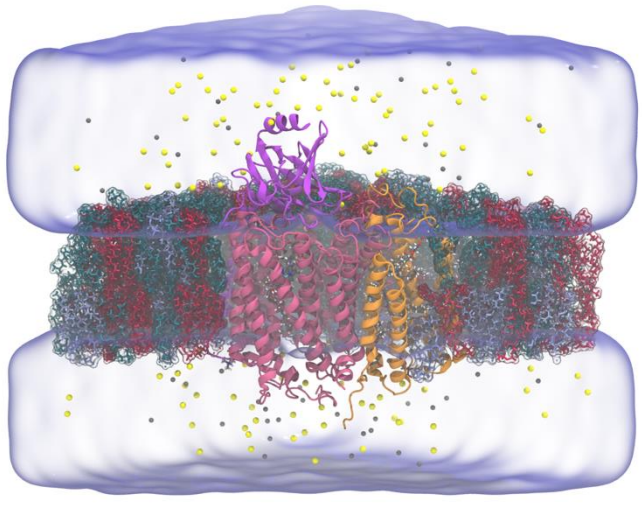


Figure 2: The initial structure of the reduced BHC*c*O (PDBID: 5B1B) embedded in a membrane and solvated, with ions for neutralization. The system here only includes the three first subunits of BHC*c*O (A,B,C) which correspond to the core reactive regions of the protein.

The calculations on GWMPC were regular MD simulations of three subunits of BHC*c*O in the oxidized (PDBID: 5B1A) form, and reduced (PDBID: 5B1B) form, and five subunits of the reduced form of BHC*c*O (PDBID: 5B1B). In all cases the protein was embedded in a lipid membrane with a composition mimicking that of the inner mitochondrial membrane, which were then further solvated in water and had Potassium ions added to it for neutralization of the system. The final systems contained around 300,000 atoms, and is shown in figure 2.

The constant force pulling simulations were mainly carried out using the BWMPC resources. The initial structures for the calculations were chosen as the structure after the first 300 ns regular MD simulation for all three systems mentioned in the previous paragraph.

Similar for all the MD simulations, both with and without constant force pulling, is that they used the Charmm36m forcefield for lipids, protein and ions. The force field for the redox active sites were adapted from the work of Johansson et al [6]. The systems were simulated with periodic boundary conditions. A timestep of 2 fs was used, with the velocity Verlet integrator. The particle mesh Ewald method was used for long range electrostatics, while the direct nonbonding interactions interactions were cut off at 12.5 Å, with a switching function used between 10 Å and 12.5 Å to reduce the interactions smoothly to zero. The Langevin thermostat was used with a temperature of 310.25 K and a pressure of 1bar.

3. Result

The regular MD trajectories were analyzed using the Hole [7] and VMD [8] programs, to elucidate the average radius of the H pathway and the water density respectively. VMD was furthermore used to generate the graphics depicting the protein and simulated system in this report. In order to have a series of reference points along the H pathway, the center of mass of a select number of heavy atoms

lining the pathway has been used to generate 5 points inside the proposed H pathway as depicted in figure 3. The points are around 7-9 Å apart and their Z-coordinate remain stable, varying around 1-2 Å, during the simulation.

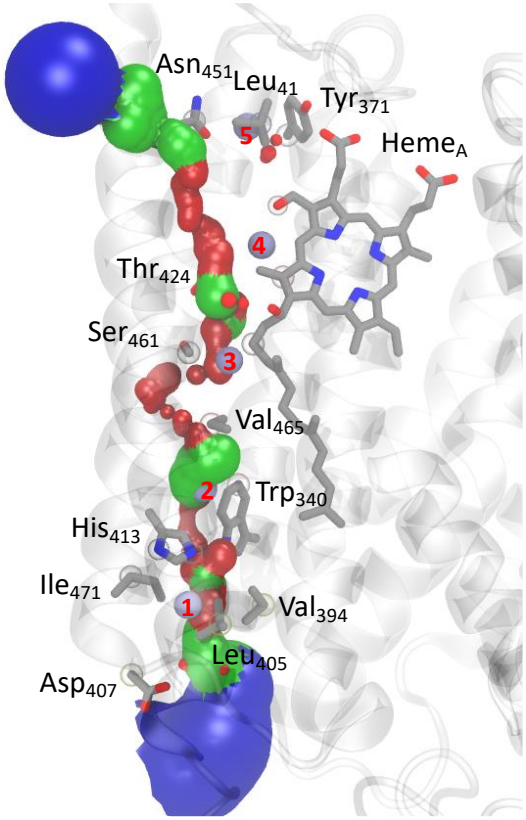


Figure 3: The H pathway, colored red – green – blue depending on its radius, generated by the Hole program [7], and the five points, indicated by grey balls and red numbers, defined by the center of mass of several heavy atoms surrounding the H pathway.

The Hole [7] program's output radius of the H pathway as a function of the Z-coordinate, shown in figure 4 for the reduced protein, are generated from trying to find the pore the protein structure every nanosecond of simulation time. When compared to aquaporin, with a radius of ~1.2 Å, we find that the pathway in some places have an average radius of ~0.8 Å. It is therefore not likely that the pathway can contain water molecules in some of its narrowest sections. This is also found for the two other systems investigated in this report. This fact can of course be an effect of water not being inside the pathway to make space between the residues. Therefore constant force pulling might possibly expand the radius of the H pathway by introducing water molecules to the narrow parts of the pathway.

Turning to the water contents of the H pathway we find in agreement with the study of Sharma et al [4] that the lower part of the pathway have a very low density of water. The water density as a function of Z-coordinate near the H pathway can be seen in figure 5 for MD simulation of the reduced protein with three subunits. Especially between point 2 and 3 there is a distinct lack of water. This indicates that the hydration of especially this part of the protein is problematic, and this region will therefore be the target of our constant force pulling simulations. The result of the water density analysis is similar for the oxidized and five subunit systems, where there is also a lack of water between point 2 and 3.

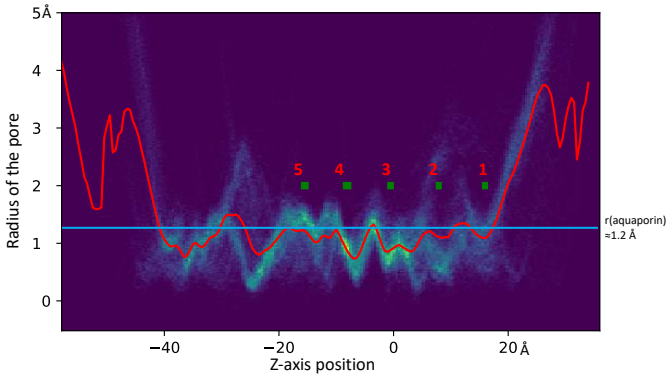


Figure 4: The average radius (red line) and a heatmap of the radii of the H pathway from the simulation of the three subunits of the reduced form of the protein (PDBID: 5B1B) sampled every nanosecond. The radius of the pore in aquaporin are used as a reference, as this protein is known to transport water molecules through its channel. The green boxes with corresponding red numbers are the Z-positions of the five points shown in figure 3.

The constant force pulling simulations are meant to calculate the rate of moving one water molecule between two of the points shown in figure 3. The biased rates can then be used to fit to a functional form which can be derived from the Smoluchowski Diffusion equation under the assumption of a cubic form of the potential energy barrier [9]. This

expression along with an illustration of the unbiased versus biased simulation are given in figure 6. We need a number of simulations for each individual magnitude of the force to determine the average biased rate as shown in figure 7, as the process of a water molecule moving between two points remains stochastic, while being significantly sped up by applying the constant force on the water molecule.

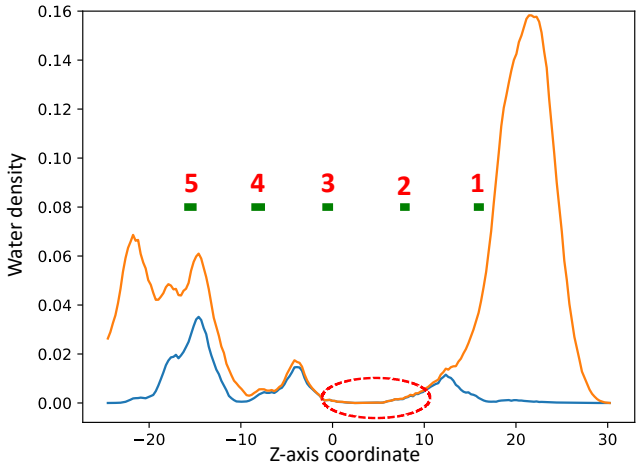
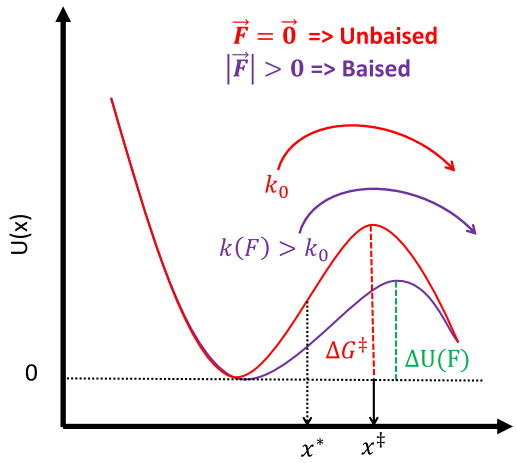


Figure 5: The water density in the H pathway of the simulated reduced state with three subunits (PDBID: 5B1B). The orange line uses the Z-coordinates of all water molecules within 10 Å of the five points defined in figure 3, whereas the blue line has been obtained by selecting water molecules using an algorithm that allows water to attach and detach from the five points based on how close or far it is from a given point. The red circle indicates the region where the density is almost zero. The green boxes with corresponding red numbers denote the average Z-position of the five points shown in figure 3.

The unbiased free energies and rates are given in table 1 for the reduced and oxidized form of BHC*c*O including three subunits. In all cases it is found that the free energy barrier for moving a water molecule between point 2 and 3 are too high to be feasible under normal temperature conditions. The free energy barrier does seem to drop when the protein changes to the reduced state, however it remains too high for normal thermal fluctuations to readily cause water to enter the area which was found to be missing water density in figure 5. Considering the hydrophobic nature of the residue found in this region, it is not surprising that a water wire is unlikely to form. We therefore find that we agree with the study of Sharma et al [4], which suggested that there is not enough water in the lower parts of the pathway to support proton transfer in either reduced or oxidized states of BHC*c*O.



$$k(F) = k_0\left(1-\frac{2x^{\ddagger}}{3\Delta G^{\ddagger}}F\right)^{\frac{1}{2}}\exp\left[\Delta G^{\ddagger}\left(1-\left(1-\frac{2x^{\ddagger}}{3\Delta G^{\ddagger}}F\right)^{\left(\frac{3}{2}\right)}\right)\right]$$

Figure 6: The two potential curves above show the biased and unbiased case with a red and purple line respectively. When we apply a force the barrier becomes smaller, and the water molecule, initially stuck in the potential minimum, will therefore faster transfer to the region right of the barrier. The equation below the figure gives the biased transfer rate as a function the applied force depending on the unbiased rate, free energy barrier and location of the barrier.

In the case of transport between point 1 and 2 in figure 3, it is found that the free energy barrier is significantly lower for the Oxidized state and might even allow for relatively fast water dynamics around His413. The reason for this might be that there is already a water molecule above His413 in the starting structure of reduced state, while there is

none in the oxidized state. This might make the barrier for adding more water in the reduced protein larger, explaining the very low barrier for the oxidized state. To further investigate this effect, we are currently attempting constant force pulling for the reduced state of the protein with five subunits included. The stating structure for this simulation does not include any water above His413, and can therefore show if the low free energy barrier are only found for the oxidized protein or also can occur in the reduced state.

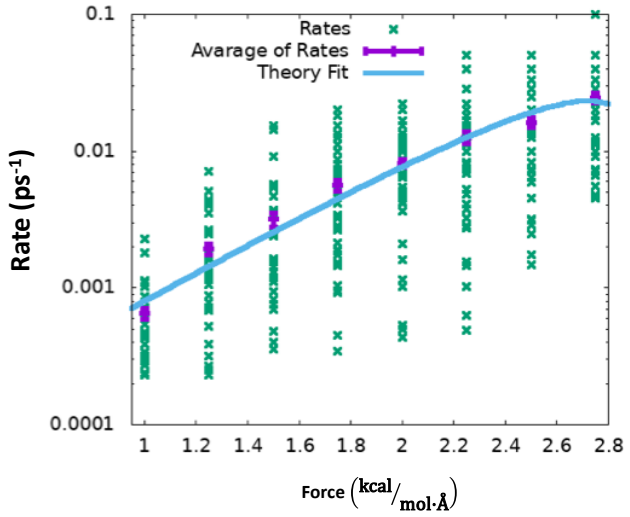


Figure 7: Fitting of the data from the pulling from point 1 to 2, see figure 3, in the reduced form of BHC*c*O (PDBID: 5B1B). The data are fit to the functional form given in figure 6.

4. Conclusion

The high free energy barriers suggest that the hydration of the lower parts of the H pathway are highly unlikely. We can therefore conclude that we do not find any evidence for the creation of a water wire in neither the reduced nor the oxidized form of the protein. The experimentally suggested reactions mechanisms involving the H pathway are therefore not supported both by the work of Sharma et al [4] and the constant force pulling simulation results from this work. Making it more likely that the proton transport in BHC*c*O happens in the D or K pathway, as suggested by many other theoretical studies.

5. Schedule and prospect for the future

We expect that the data collected in this project will form the basis of a future publication on the feasibility of water being present in the lower parts of the H pathway of BHC*c*O. Currently there are still some simulations that needs to be completed, and additional analysis of the trajectories which needs to be undertaken. We do expect that this will be done by the end of this fiscal year using local computational resources, and that the writing can begin in the beginning of the next fiscal year.

Transition	1 => 2	2 => 3	3 => 2
	Reduced BHC*c*O (PDBID: 5B1B)		
k_0 (ns^{-1})	$2.7 \cdot 10^{-2}$	-	$1.1 \cdot 10^{-2}$
ΔG (kcal/mol*Å)	7.98	-	10.8
	Oxidized BHCcO (PDBID: 5B1A)		
k_0 (ns^{-1})	$8.9 \cdot 10^{-1}$	$1.2 \cdot 10^{-1}$	$1.4 \cdot 10^{-1}$
ΔG (kcal/mol*Å)	2.91	8.93	7.07

Table 1: The unbiased water molecule transfer rates and free energy barriers found by fitting the biased rates found in our simulations, see figure 5 for the analytical form and figure 6 for an example of the fitting procedure.

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[2] S. Yoshikawa et al., Science 280, 1723-1729 (1998)

[3] P. R. Rich and A. Marechal, J. R. Soc. Interface 10, 20130183 (2013)

[4] V. Sharma et al, PNAS 114, 10339-10348 (2017)

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[7] O. S. Smart et al, Biophys. J. 65, 2455 (1993)

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[9] O. K. Dudko et al, Phys. Rev. Lett. 96, 108101 (2006)

Fiscal Year 2018 List of Publications Resulting from the Use of the supercomputer

[Poster presentation]

Thomsen B, Sugita Y "Constant Force Pulling Simulations for the Hydration of the H channel of Cytochrome c Oxidase" Danish Chemical Society Annual Meeting 2018, August 23rd.

Thomsen B, Sugita Y "Computational Investigation of the Hydration of the H Channel of Bovine Heart Cytochrome c Oxidase" 20th European Bioenergetics Conference, August 25th.