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Review of the doctoral dissertation of M. Sc. Maria Bzówka on "Analysis of molecular aspects of proteins regulation considering water molecules as potential mediator in intermolecular interactions".

The subject of research described in the dissertation concerns the development of different tools and also their application for the analysis of water molecules, tunnels and pathways in different biomolecules, including different enzymes (proteases, epoxide hydrolases), and toll-like receptors. The tools developed in the thesis and the performed analysis is of importance for drug-discovery, and also protein engineering and design.

The doctoral thesis consists of an introduction that focuses on the importance of the subject under study, followed by an overview of the developed strategies for evaluating water and its role in biomolecular systems. The introduction is well-written, easy to read and provides the key concepts and references for introducing the subject of the thesis. There are no figures included in this first part of the thesis, and probably a summary figure including some of the available strategies to study water content in proteins (both experimentally and computationally) could be useful to put the reader in context.

In the following sections, details about the motivation of the thesis and the computational details and conditions of computational experiments are provided. The aims of the thesis are also nicely detailed and specified, and maybe the main general goal of the thesis could be also included before the detailed sub-objectives are described. I like the connection of each subobjective with a particular paper included in the thesis. It should be mentioned that the thesis is based on 9 different published papers, plus one preprint publication. The list of papers is long, and all are of very high quality. In chapter 2 (Aims of the thesis), the term intramolecular void is included, which I believe it would be good to previously define it (in the introduction).

In chapter 3, the results obtained in the different papers are discussed and summarized. In section 3.1, the review paper about the application of water molecules for analysing different properties of macromolecules is included, as well as paper 2 based on the development of the new version of AQUADUCT 1.0. In my opinion it would be good to include a clear description of the terms used along the thesis: tunnel, channel, voids, cavities (probably in the introduction as mentioned before), and maybe adding a workflow of the software AQUA-DUCT, especially regarding the valve and pond drivers.

In section 3.2, the application of water and cosolvent molecules for drug design is described. In particular, paper 3 based on evaluating the differences in water content, tunnels in the SARS-CoV and SARS-Cov-2 proteases is described. Several computational techniques are applied to characterize and evaluate the differences between the two proteases in the presence and absence of inhibitor. It should be mentioned that this study opened the possibility to collaborate with the University of Basel, and indeed paper 4 is included, which focuses on assessing the selectivity of protease inhibitors against SARS-CoV-2. In this section, the concept off-target is also included, which should be also described. This section also includes paper 5, that focuses on the analysis of all deposited HsEH-ligand complexes to get some insights into the binding of inhibitors and improve the drug design process. This again another interesting study done in collaboration with UC Davis, and it should be highlighted that some of the new inhibitors found led to a

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patent application. In page 47, in the second paragraph there is a minor typo "indicted" that should be corrected.

In section 3.3, some applications of the analysis of water molecules in protein regulation and engineering are provided. This includes paper 6 in which structure-function relationships between soluble epoxide hydrolases and their tunnel network are developed. In this section, I found differences in capitalizing the different living organisms: Bacillus megaterium EH is abbreviated as bmEH, whereas Solanum tuberosum as StEH. This should be corrected for consistency. Paper 7 focuses on the comparison of geometry-based methods (CAVER) versus small-molecule tracking (AQUA-DUCT) for identifying tunnels and is based on the same set of epoxide hydrolases used in paper 6. Finally, in paper 8 the evolution of tunnels in the alpha/beta-hydrolase fold is evaluated. For me this is a very important and interesting study, as they find and characterize tunnels probably reminiscent of ancestral variants. One of the descriptors used is entropy as defined in the BALCONY package. I think it would be convenient to add some additional information on how entropy is computed.

Finally in section 3.4 the role of water in Toll-like receptors (TLRs) is analysed. TLRs are very challenging to computationally characterize as described in the review paper 9. It is also interesting to note that the role of water molecules on the entire reaction cycle was also computationally evaluated (preprint 1). Although the QM and QM/MM calculations were not done by the candidate, I still think it would be good to include a schematic drawing of the reaction mechanism to better follow the description of the methodology and the subsequent steps based on MD simulations at the different intermediates, reactant complex and product.

In general, many of the figures included in the results section have pretty low resolution, I think it would be better to change them for a higher resolution one. This includes figures: 4, 5, 6, 7, and 8.

The last chapter of the thesis includes the most important conclusions withdrawn from the thesis as well as the future perspectives.

In summary, I conclude that the submitted dissertation meets the requirements set out in the Act on academic degrees and academic title and on degrees and title in the field of art of March 14, 2003 (Journal of Laws 2003 No. 65, item 595, as amended). Therefore, in my opinion, M. Sc. Maria Bzówka can be admitted to further procedures necessary to obtain the PhD degree. Moreover, taking into account: the fact that Maria Bzówka is a co-author of 9 publications published in highly ranked, internationally recognised journals, she has participated in multiple international conferences and workshops, she has conducted all analysis included in this doctoral dissertation and the high importance of the provided data of importance for the protein engineering and drug discovery fields, ***I recommend this thesis for being awarded.***

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