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**Review of Doctoral Thesis**
**Mgr. inż. Aravind Selvaram Thirunavukarasu**

**Title:**
**Role of Transient Tunnels in Function of Enzymes with Buried Active Sites**

The work submitted for review presents and discusses the results of research conducted by M.Sc. engineer Aravind Selvaram Thirunavukarasu at the Faculty of Chemistry, University of Adam Mickiewicz in Poznań. The supervisor of the thesis is Dr Hab. Jan Brezovsky, prof. AMU. The work is presented in the form of a collection of three published and thematically related scientific articles. This is in line with the general requirements of Art. 187 of the Act dated July 20, 2018 Law on Higher Education and Science, as amended (Journal of Laws of 2023, item 742). The act does not define what types of articles there are and where they should be published, but for the record, I would like to state that two of the three articles were published only as preprints in bioRxiv, which means they were not previously subjected to the peer review process. They also do not have IF or MEiN scores. Typically, such a collection of published articles in a doctoral thesis is preceded by an introduction. In this case, such an introduction was also prepared by the PhD candidate. It included a summary of doctoral thesis and conclusions and future perspectives. Unfortunately, this introduction did not contain an explicit presentation of the purpose of the work, and as a reviewer I can only guess that the purpose was or could have been to learn about the properties of water tunnels in globular proteins, and in particular in haloalkane dehydrogenase. Nevertheless precise objectives of the doctoral thesis is needed. Research on these properties was conducted using molecular modeling methods, including the TransportTools software developed by the Promoter's team. The introduction only contains a discussion of the results of the work and the lack of a formulated purpose of the work seriously hampers the assessment of the doctoral candidate's achievements. In fact, it is not known whether the set goal was achieved.

However, returning to the content of the PhD thesis, it should be emphasized that the subject of the work is interesting, because so far little research has been devoted to water tunnels in proteins and their functioning. The methodological approach used in the research presented by the PhD candidate and presented in the publications is quite innovative. The authors of the publication use the results from molecular dynamics simulations of water tunnels, which include water transport in these tunnels. These results are then analysed using

CAVER and AQUA-DUCT software to generate transport routes. A new solution, which should be considered as a scientific novelty, is to enable the analysis of data from these two programs for many trajectories, which allows for a more consistent analysis of the properties and functioning of water tunnels. Such an analysis takes into account data from all MD simulations performed simultaneously. The tool for this analysis is the TransportTools program. The doctoral student, as one of the co-authors, tested this program for one of the protein systems, i.e. haloalkane dehydrogenase protein. Other authors from these papers tested other protein systems. Additionally, the PhD candidate and other co-authors tested various water models (TIP3P, TIP4P-Ew, OPC) to determine how these types of water behave in water tunnels. In particular, these tests again included haloalkane dehydrogenase.

Even though the presented study contains statements from co-authors of published works, it is still not entirely possible to clearly determine what should be considered as the PhD student's contribution and his scientific achievement. Therefore, I will try to evaluate the overall achievements presented in the doctoral thesis related to haloalkane dehydrogenase. I would also like to mention here that the PhD candidate, presenting his PhD thesis in the form of a collection of multi-authored publications in which many protein systems were studied, made writing the text much easier for him. At the same time, it made the assessment of such a study extremely difficult for the reviewer, because many research results are not even included in the works themselves, but in supplementary materials that the reviewer had to download from the Internet. In this case, it might be more reasonable to collect all the PhD candidate's achievements in the form of a classic work, which would contain both data from publications and supplements, only those that were authored by the PhD candidate. Such a study would be even more justified because two of these works, as I have already mentioned, were published only as preprints.

Assessing the topic of the work, it should be stated that the research conducted for many systems, including haloalkane dehydrogenase, brings a lot of interesting information about the transport properties of water channels in proteins. By performing MD simulations and using adaptive sampling followed by CAVER and AQUA-DUCT analysis and TransportTools analysis it was possible to collect new data about studied systems. Obtained data are solid contribution to understanding of conformation of these systems and water movement as well as transport pathways in the tunnel system in enzymes. In particular interesting was a finding that even narrow pathways still can transport water but to do this an increase number of hydrogen bonds between water molecules and aminoacids residues is required.

The work arrangement, as I have already mentioned, is unconventional. However, the Introduction part and presented study, despite the lack of the purpose of the work, is quite well written and presents a kind of theoretical introduction and discussion of the results contained in the three works. These results include studies on water transport within haloalkane dehydrogenase tunnels and concern various water models used in these studies. In addition to the final conclusions, the summary paragraph also includes future perspectives.

Due to the reviewer's obligation, I must refer to various types of weaknesses of the work or generally to problems with its structure and content.

General comments:

1. Due to the form of the work and its multi-threaded nature as well as the participation of many authors in the project, as part of the defence, the PhD candidate should present a detailed description in bullet points of what his authorship and achievement actually were. This is due to the fact that some of the co-authors' statements are still not clear to catch this information.

2. The doctoral student should present what the purpose of the work was, and therefore answer the question whether it was achieved and to what extent.
3. There is no firm conclusion in the work as to how the results obtained by the doctoral student translate into expanding knowledge in the field of research on protein water tunnels. The same applies to research on water models. The statement I quote "These results highlight the critical role of the chosen water model in simulating complex enzymatic actions, informing enzyme design strategies, and predicting drug residence times" is very general. The PhD student does not indicate which water models should be used and for what purpose. However, the statement that the TIP3P model is the most mobile is not groundbreaking information because this water model has the highest diffusion coefficient among many models and the fact of this mobility has already been discussed many times in literature.

Detailed notes:
1. Page 17 The last sentence regarding mutations should be expanded because it is not clear what is meant in connection with the citation of ref. 44.
2. Page 19 Building MSM models should be better described.
3. Page 22 The names new_A and new_B do not match the names in Figure 6B, D
4. Page 28 In the legend of Fig. 11 there is no reference to where this data come from.
5. Publication no. 2, page 5. It is not known why the reference to pH 8.5 was used when preparing the Hal model. Ref. 36 concerns the free enzyme outside the cell and gives a pH range of 8-9.5. Rather, the enzyme in the cell operates in the pH range of 7.4 so why 8.5 was selected?
6. Publication no. 2, page 6. Why was the time step in MD as much as 4 fs, and not the classic one, e.g. 2 fs?
7. Publication no. 2, page 7. The use of short time simulations of 100-200ns followed by HT MD in which a limited number of amino acid residues were defined as mobile may not fully reproduce reality. What about substantial conformational changes in large protein fragments that may significantly change the diameter of water tunnels? This events can occur only when longer MD simulations are run.
8. Publication no. 3 Figure 1c. There is no literature reference to experimental data in the legend.
9. Publication no. 3 Material and methods. The proposed approach described in this paper gives a good static picture, but does it accurately reflect the dynamics of the investigated tunnels? In particular, is it possible, using the approach used, to notice, for example, that when a given tunnel opens at a given moment, another one closes? Can such positive or negative correlations be observed and described using the methodological approach applied?
10. Publication no. 3 Results and discussion. Figures S1 and S2 have not clear description. The X-axis represents the simulation number, and meanwhile on the graph we have some distributions. The legend doesn't explain what it is.

Statements of co-authors of the publication.
Although the resolution of the Adam Mickiewicz University Senate allows for collecting statements from at least 4 co-authors when the work has more than 5 authors, in the case of a doctoral thesis consisting of publications, in my opinion, such an approach is not entirely appropriate. It seems logical that as the number of contributors increases, the doctoral candidate's share decreases and all statements are helpful in assessing his or her contribution in this more complex PhD thesis. Just to note, In the case of the first, and actually the most important work, because it was published in a recognizable journal, there are no statements from two co-authors.

Summary:
Despite my many critical remarks presented above, I think that the subject of the work is interesting and I believe that the doctoral candidate's achievement is most likely significant, provided that he presents the purpose of the work and clearly identifies his contribution to the resulting publications, i.e. defines his scientific achievement. Therefore, I am applying to the Discipline Council of Biological Sciences of Adam Mickiewicz University to admit Mr. Mgr. engineer Aravind Selvaram Thirunavukarasu to the next stages of the doctoral process.

Maciej Bagiński