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Evaluation report of the thesis
Action at a Distance in Arginine Repressor and the Store-Operated Calcium Channel Orai: Molecular Modeling and Simulations
submitted by *Saurabh Kumar Pandey*

The thesis of Saurabh Kumar Pandey is based by five publications (four journal articles and one book chapter). They are equipped by an introduction, materials and methods section and results overview. This accompanying text makes it clearly possible to assess the role and contribution of the applicant to each publication.

The applicant had a complicated task to write the introduction because the model proteins come from completely different organisms. There are few sentences where the author switched suddenly from eukaryotes to prokaryotes, but in general he did an excellent job. The explanation of allostery is comprehensive. The role of allostery in drug design is briefly mentioned. At this point I was missing mentioning the fact that the world "allosteric" is often used in two meanings. It is either used in the meaning used by the applicant. For example, G protein-coupled receptors are allosteric in the sense used by the applicant because binding of a ligand at one side of the receptor modifies binding of G protein on the opposite side. However, many pharmacologists call binding of a ligand to its canonical binding site "orthosteric" and only ligands binding to non-canonical sites are called "allosteric". I believe the author is aware of this but some cited studies are likely to use the word "allosteric" in the second meaning.

At page 5 the author states that negative cooperativity can be explained by KNF but not MWC model and that for explanation of ArgR both models were used. I understand that ArgR is negatively cooperative (I missed some figure from the literature to show this) so how it could be explained by MWC model?

The thesis is nicely illustrated. In illustrations of ArgR I was missing the information which trimer of the hexamer was used in fitting (e.g. Figure 1.3 and 1.4). With the naked eye it looks like the bottom monomer was the one used in fitting. This information would be also useful in other calculations used in articles, namely RMSD and PCA.

As far as I understood, the structural basis of STIM interaction with Orai is not known. It is very well explained, nevertheless some schematic illustration would be useful.

The Orai sub-project presents two interesting studies, one on the role of its N-terminus and loop2 and one on cholesterol binding. As far as I was able to trace, the role of the N-terminus and loop2 was studied without taking into the account the role of cholesterol, at least in the molecular modeling part. Would the message of the first Orai study (in *J. Biol. Chem.*) change in the light of findings of the second study (in *Sci. Signal.*)?

Cholesterol has been known for a long time to modulate function of GPCRs. The study showing that Orai is also regulated by cholesterol is very interesting. Does the applicant believe that the cholesterol binding site is druggable and its modulation can be therapeutically useful? There are many people consuming cholesterol-lowering drugs worldwide. Is there any hint that pharmacologically induced cholesterol lowering may have an effect on Orai function, either negative or positive for the patient?

Finally, I have a general question related to allostery and cooperativity. It is clear why for example binding of oxygen to hemoglobin is positively cooperative, simply because oxygen can almost fully load hemoglobin in lungs and can be almost fully released in low oxygen tissues. But what is the point of negative cooperativity? Is this somehow explained? As far as my knowledge goes, simple decrease of

Hill's coefficient bellow 1 does not increase affinity towards the ligand at low concentrations. I would greatly appreciate to hear the opinion of the applicant on this.

As already mentioned, the thesis is nicely illustrated. It is also nicely written. One minor issue is inconsistency in writing Latin names of microorganisms (use of italics). Also Latin names of higher taxonomic units should be in italics. This minor point does not reduce the overall quality of the thesis.

In conclusion, the thesis and presented high quality publications significantly contribute to our knowledge of allostery and ion channels. They clearly show that the applicant is an expert in molecular modeling and is capable to conduct independent scientific research. **Therefore I strongly recommend University of South Bohemia in České Budějovice to accept this thesis**.

Vojtěch Spiwok

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November 31th, 2018

Morteza Khabiri, Ph.D.
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Dear Committee Member,

Review of the PhD thesis

"Action at a Distance in Arginine Repressor and the Store-operated Calcium Channel Orai: Molecular Modeling and Simulations"
submitted by
Saurabh Kumar Pandey

Saurabh Kumar Pandey used computational methods as investigative tool to look at the phenomenon of allostery. The PhD work investigate the allosteric effect into two different systems: a) arginine repressor and b) the human Orai channel. His work resulted in contributions to 5 publications: one with Saurabh Kumar Pandey as first author, has been published by *Journal of Molecular Modeling* while the other is a book chapter in a book entitled as "Methods and Principles in Medicinal Chemistry". Saurabh's focuses in the first part of his PhD thesis on the molecular origin of the transition of ArgR in two different states: apo and holo. Arginine repressor protein is a master regulatory molecule that modulates arginine biosynthesis and catabolism in bacteria in response to intracellular arginine levels. Saurabh was studied the allosteric effects of L-arginine binding on structure and dynamics of ArgR. He found that the rotational state of ArgR in both B. subtilis and in E. coli is similar. In both biological systems the binding of L-argenine start the rotational motion that induce structural changes toward most favorable DNA-binding states.

The effect of cholesterol on the structure of human Ori1 channel and compare the Ori1 and Ori3 isoform channels are the target of the second part of Saurabh Kumar Pandey 's PhD. Orai channels are responsible for entry of calcium ion in the cell in response to Ca2+ depletion in the endoplasmic reticulum. He found that cholesterol has modulatory role in regulating Orai1 channel function via controlling the pore size. Moreover, he compared highly conserved regions called extended transmembrane Orai N-terminus between two Ori channel isoforms. He discovered that N-terminus and loop2 are required to store-operated function of Orai channel.

During the course of his PhD, Saurabh Kumar Pandey gained a profound knowledge of computational techniques and used them to investigate molecular interactions between proteins and with small molecules. Linking macroscopic experimental observations with microscopic details at the

atomic level is difficult. The PhD candidate Saurabh Kumar Pandey demonstrated in his PhD thesis to be able to successfully carry out these tasks. Further discussion of the methodological aspects relevant for the project as well as the pitfalls and their solutions would be welcome.

1- Allostery is best described by a series of free energy landscape and the corresponding energy levels. Cite specific examples of free energy changes that cause allosteric effect in your systems?

2- Autodock-vina was used to create a model of the complex consisting of the Ori1 channel and the cholesterol. Which principles do these docking approaches follow and what are the strengths and weakness of available approaches?

3- Molecular dynamics simulations is a powerful method that can be used to explore the energy hypersurface of proteins, complexes and to measure the energetics involved. A requirement for any analysis is ergodic sampling. A discussion of what ergodicity is and how it can be checked for would be welcome.

Saurabh Kumar Pandey has published five papers and one book chapter, two of them as first author. This underlines here outstanding achievements. I can therefore assess the PhD thesis submitted by Saurabh Kumar Pandey as excellent and recommend Saurabh Kumar Pandey for the admission to the PhD defense.

Best regards

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Report on Ph.D. thesis
of
Saurabh Kumar Pandey

"Action at a Distance in Arginine Repressor and the Store-operated Calcium Channel Orai: Molecular Modeling and Simulations"

The dissertation of Saurabh Kumar Pandey is a valuable contribution to the study of phenomenon of allostery which is important both in basic research and in rational drug design. The whole work represents very suitable combination of various computational methods used to shed light on this subject in the context of arginine repressors and human Orai channels.

The first L-Arg binds to hexameric arginine repressor (ArgR) with ~100-fold stronger affinity than the remaining five (it means that there is negative cooperativity among the six structurally identical binding sites). The mechanism by which binding of L-arginine to the core oligomerization domain of ArgR allosterically affects the affinity of peripheral DNA-binding domains to DNA remains unclear.

i) Therefore, ArgR of E. coli was studied by 100 ns molecular dynamics simulations. A rotational shift between trimers followed by rotational oscillations occurred only when L-Args were absent. Nevertheless, binding competent states were occasionally sampled and docking of just one L-Arg resulted in a holoprotein-like conformational distribution of ArgR in subsequent MD simulations.

ii) Further, B. subtilis ArgR was studied by 2us MD simulations. The rotational behavior of trimers was very similar despite of very different amino acids that mediate crucial mutual interactions. It indicates functional importance of these motions. It seems that binding of L-Arg stimulates rotational shift of ArgR trimers enabling that N-domains of ArgR can sample DNA-binding states.

Next, structural-functional properties of Orai channels (mutants of which are associated with immunological diseases, muscular dystrophy etc.) were investigated.

iii) MD simulations suggested that in the non-functional Orai1 N-truncation mutant the remaining N-terminal portion formed inhibitory interactions with the Orai1-loop2 region, but not with Orai3-loop2. Indeed, such a loop 2 swap restored activation of the N-truncation Orai1 mutants.

iv) Orai1 senses the amount of cholesterol in the plasma membrane. In fact, cholesterol inhibits Orai1 activity. Single point mutations in the Orai1 N terminus that were expected to abolish cholesterol binding enhanced Orai currents to a similar extent as did cholesterol depletion.

v) Subsequent docking of cholesterol to full-length human Orai1 did indeed reveal binding. However, the cholesterol binding was not found at the helical ETON region in the Orai1 N-terminus. Instead, it occurred at a cholesterol binding pocket located between TM2, TM3 and TM4 from an Orai1 dimer. This indicates that cholesterol binding to the Orai1 TM2/3/4 domains is probably allosterically affected by these point mutations in the Orai1 N-terminus.

vi) The last contribution attached to the thesis is a comprehensive, dense and well-organized review of ion channel MD simulations.

It is evident without any doubts that Saurabh Kumar Pandey masters wide range of modeling techniques (sequence and phylogenetic analysis, homology modeling, docking, force field parametrization, molecular dynamics simulations etc.) using software packages GROMACS and Yasara. He knows to suitably apply them to get valuable data that can be verified by an experiment.

The scientific results are of major interest and substantially contribute to the knowledge of phenomenon of allostery. There are enclosed four papers (including recent J. Biol. Chem. (2018) with IF ~ 4.3 and 3 citations and Sci. Sig. (2016) with IF ~ 7.4 and 30 citations) and one

manuscript. Another paper could appear soon, if we take into account the unpublished data about interactions between channel Orai and cholesterol.

In my opinion, the work certainly fulfills the requirements for Ph.D. thesis, and I recommend it for the defense.

Prague 30th November 2018

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QUESTIONS:

I) Explain basic models of allosteric regulation using schematic images.

II) Discuss the results obtained in your manuscript "Allosteric activation of Arginine repressor protein by L-arginine" in the context of the crystal structures 3FHZ, 3LAJ, 3LAP (1, 2). What is the key structural change in N-domains that allows DNA binding?

III) In (3), positions -77 to -25 in the promoter region were found to be important for the DNA binding of ArgR. Is this binding site of ArgR common in all bacteria?

IV) Based on the crystal structure 4YLN of bacterial RNAP (4), deduce with which subunit of RNAP will ArgR compete.

V) Schematic sketches in Figure 1 (5) and Figure 6 (6) can be now - after one decade - largely replaced by crystal structures (6-11). Could we produce a MD simulation of the whole Orai-STIM complex?

VI) Are very recent Orai structures 6BBQ, 6BBF, 6BBH, 6BBI (11) shedding a new light on conclusions of your Paper 3?

VII) Could these structures change conclusions of your Paper 4?

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Figure12