Uniwersytet Gdański | Związek Uczelni Fahrenheita CHEMIA UG

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Review of the doctoral dissertation

of Mr. Thiliban Manivarma, M.Sc.

entitled "Regulation of Ferroptosis through 15LOX-1/PEBP1 Complex – Computational Modeling at Molecular Level"

Apoptosis (from classical Greek ἀπόπτωσις, falling off, disappearance, also ἀποπτύω, I spit out) is a collective term for a plethora of processes used by living organisms to dispose of aged and, thus, dysfunctional or potentially dysfunctional cells in a controlled way (as opposed to necrosis, which is unwanted and uncontrolled cell death, usually extending to a part of a tissue). One of those is ferroptosis, which is triggered by ferric-ion catalyzed lipid peroxidation. This process initiates membrane destruction and, ultimately, cell lysis. This important mechanism was discovered quite recently (in 2012). The key enzymes involved are lipoxygenases (LOX). Despite the significance of ferroptosis, our knowledge of its mechanism is still incomplete.

The Ph.D. research of Mr. Thiliban Manivarma was devoted to theoretical investigation of the molecular mechanism of the ferroptosis catalyzed by the complex of 15-lipoxygenase-1 (15-LOX-1) and phosphatidylethanoloamine-binding protein (PEPB1). Mr. Manivarma did his Ph.D. work under the supervision of Professor Karolina Mikulska-Rumińska, a world-recognized scientist of ferroptosis research. The theoretical results were validated by the experiments carried out by the collaborating experimental group.

The Ph.D. thesis is based on 3 papers, referred to as papers A, B, and C, respectively. The Candidate is the first author of Papers A and C. Papers A and B have already been published in excellent international scientific journal with high impact factors (*Free Radicals in Biology and Medicine* and *Bioinformatics*, respectively), while paper C (submitted to *Redox Biology*) has not appeared by the time of completing this review. The preamble of the thesis consists of Acknowledgements, Table of Contents, List of Abbreviations and Abstract (in English and in Polish, respectively). The body of the thesis consists of Introduction (section 1), Publications constituting and dissertation (section 2), Author contribution statement (section 3), The aims of the Ph.D. project (section 4), References (section 5), Papers (section 6), Co-author contribution statement (section 7), Summary and future prospects (section 8), and Appendix (section 9). Apart from the Polish version of the Abstract, the whole thesis is written in English.

As follows from the statements of the Candidate (sections 3) and co-authors (section 7), Mr. Manivarma was the leading contributor and did the theoretical research pertaining to papers A

and C, while in paper B, which describes the newly developed WatFinder software, he did the testing of the software. Therefore, his contributions to the papers has undoubtedly entitled him to include all of them in his dissertation. Papers A and B passed the peer-review process before they were published and, therefore, their merit is undisputable. Consequently, when reviewing the material of the thesis I scrutinized mainly paper C, as well as assessed whether the accomplished research is sufficient for a Ph.D. thesis.

The specific aims of Mr. Manivarma's Ph.D. work listed in section 4 can be summarized as (i) determining the dynamics and structural changes of the 15LOX-1PEB1 complex in the presence of the membrane and substrate (ii) evaluating the impact of PEBP1 mutations of interfacial residues on the dynamics of the complex, (iii) identifying oxygen diffusion pathways and proposing the mechanisms of peroxidation. The Candidate used all-atom explicit-solvent molecular dynamics simulations with the CHARMM force field implemented in the NAMD software to accomplish most of the tasks. The parts of the systems studied not covered in the standard CHARMM were parameterized by using DFT.

The Introduction (section 1) consists of a succinct review of apoptosis, the description of the specific systems studied by the Candidate, and a summary of the computational methods used by the Candidate, including an outline of computation setup. In section 1.1, the history of the studies of apoptosis and a short description of this phenomenon and that of the variants of apoptosis are presented. Section 1.2 is specifically devoted to ferroptosis, including the mechanism of lipid peroxidation catalyzed by LOX and the inhibition of this process. These two sections are very well and systematically written and provide an excellent introduction into the state-of-the-art science of ferroptosis. In section 1.3, the Candidate introduces the bio-molecular system that was the object of studies of his thesis: the 15LOX-1/PEBP1 complex interacting with the lipid membrane composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanoloamine (DOPE), and 1-steraoyl-2-arachidonyl-1-phosphoethanoloamine (SAPE), the latter being the preferred substrate of the 15LOX-1/PEBP1 enzyme.

In section 1.4, the main computational methods used, namely molecular docking and molecular dynamics, are described. The methods for calculating the molecular electrostatic potential based on the Poisson-Boltzmann (PB) equation, which were used in papers A and C, should also be mentioned. The PB method is mentioned only in an unrelated context in the beginning of page 28. This section is written from the application-scientist point of view, focusing on software and models and not so much on the physical principles. Such an approach is fine in the Methods section of the papers but, in the Ph.D. thesis, the Candidate could elaborate more on the physics behind the computational methods used. Moreover, in my opinion, section 1.4 could be organized better. A clear distinction could be made between the parts corresponding to force fields, molecular dynamics as such, simulation regimes, calculation flow, etc. Finally, making a separate titled subsection to describe the specific simulation settings used by the Candidate instead of including this information at the end of section 1.4 would improve the clarity.

A comparable information and detail load should be included in each part of section 1.4. For example, a rudimentary energy expression for the force field is given in page 26 but the Newton equations of motion are not. It is even not clear how is the expression for the energy related to these equations. Algorithms for numerical integration of the equations of motion (e.g., the leap-frog algorithm) should at least be mentioned. Instead, the Candidate only refers to the SHAKE algorithm, which is part of numerical solution of the equations of motion with explicit solvent molecules (water in this case).

The basic regimes of MD simulations: constant-energy (NVE), as well as the thermostated regimes (NVT and NpT) should be discussed and not only mentioned (page 28). The Candidate should state explicitly which regime was implemented in his studies. Indirectly, from the "Langevin piston" keyword, it can be realized that his calculations were run in the the NpT regime (page 29). Besides, the "Langevin dynamics" term that the Candidate uses in page 29 strictly applies to simulations with implicit solvent only. A more appropriate term that applies to the Candidate's simulations is the "Langevin thermostat" (and barostat). Other thermostats/barostats (Anderson, Bernedsen, etc.) should also be mentioned.

The last paragraph that starts in page 27 is about energy minimization. I suppose that the Candidate used this step to relax the starting structures before running MD. The first sentence of the paragraph that states that the purpose of energy minimization is to find the global minimum configuration is wrong. Finding the global minimum is the purpose of global minimization and not any minimization. Global minimization is generally an NP-complete problem and what the Candidate describes later refers to local minimization. Also, I do not think that it was correct to mix the minimization description with correcting for protonation states; this is a completely different problem that cannot be tackled by energy minimization.

In the last paragraph of page 28, the Candidate states that the CHARMM force field parameters for bonded iron were obtained using the DFT method with the B3LYP functional and the 6-31G(d,p) basis set. It is not stated, though, what specific parameters were derived (bonded, nonbonded, charges) or what was the model system for parameterization was. Neither could I found this information in the attached papers. This information should be given and the determined parameters listed.

The results concerning the investigated mechanism of ferroptosis are described in papers A and C, while paper B reports the development of the WatFinder software, which was implemented in the research described in paper C.

In paper A, the dynamics of the 15LOX-1/PEBP1 system in the absence and presence of the lipid membrane was studied. The key issue is the opening of the interface between the two components. The initial models of the complex were constructed by using molecular docking of the experimental PEBP1 structure to the experimental 15LOX-1 structure. Two plausible models, referred to as models 1 and 3, respectively, were selected based on the results of earlier investigations. MD simulations conducted by the Candidate demonstrated that the cavity opens only when the interface gets in contact with the membrane surface. The opening enables the

extraction of a lipid molecule from the membrane to the catalytic site of the enzyme. Further MD simulations demonstrated that the cavity closes upon substrate binding. By mutational analysis the Candidate found the critical role of the P112 residue in opening and binding. The importance of P112 was confirmed by the liquid chromatography-mass spectrometry (LC-MS) experiments carried out by the collaborating experimental group, which are also reported in the paper. Especially the P112E mutation was deteriorating, over-increasing the size of the cavity between the two components. The reason for this was electrostatic repulsion resulting from the replacement of P112 with a negatively-charged glutamine residue.

In paper C, the Candidate focused on oxygen diffusion to the catalytic site. (The pages of this paper are not numbered, so I will refer to consecutive page counting in the part of my report concerning paper C.) For this purpose, 160 independent MD simulations, according to the statement the beginning of Results and Discussion, of the 15LOX-1/PEBP1 complex were carried out in the presence of 1, 2, and 15 dioxygen molecules, respectively. However, from Table 1 of the Supplementary Material it seems that there were 195 simulations. The two additional series consisted of 30 and 5 runs, respectively, in which the PEBP1 part of the complex was not present. These series are unaccounted for in the statistics presented in Supplementary Table 2. It should be noted that the results of the simulations without the PEBP1 part of the complex are presented in panel (b) of Figure 2 pertaining to the distribution of dioxygen molecules in the catalytic site and discussed in page 9 of paper C.

Of the simulation series (20 runs each) accounted for in Supplementary Table 2, 3 were carried out in the presence of substrate (SAPE) and with the membrane, 2 in the presence of SAPE and without the membrane, 1 without SAPE but with the membrane and 2 without SAPE and without the membrane. In the absence of the substrate, the dioxygen molecules were randomly distributed after MD simulations, none reaching the catalytic site, while in the presence of the substrate they explored two tunnels leading to the catalytic site, with entrances A and B, respectively, regardless of whether the oxygen molecules were initially placed near the catalytic site or in water. The substrate-induced conformational change was found to trigger oxygen access to the catalytic site. The presence of the membrane did not affect the accessibility of the catalytic site by the dioxygen molecules but affected the residence time.

In the next part of paper C, the water clusters near the active site and in the oxygen-access tunnels are analyzed. For this purpose, the Candidate used the WatFinder software, which he co-developed, and which is described in Paper B. The distributions of water molecules from both MD simulations and from the crystal structures of LOX isoforms have been analyzed. Based on this analysis, the Candidate postulated that the presence of water found near the catalytic site, as determined by this analysis, can facilitate oxygen transport, serve as the source of protons in the redox reaction, and facilitate the removal of the more polar peroxidated products. Subsequently, the analysis was extended to the clusters of dioxygen molecules. The Candidate found that one single dioxygen-molecule occupancy of the catalytic-site neighborhood is most probable but two dioxygen molecules are also present. This prediction was confirmed by the respective LC-MS analysis of the peroxidation products.

I have the following minor technical comments on Paper C:

Figure 2. Panels (a) and (b) of Figure 2 overlap. It looks like the graph representing the number of water molecules at the catalytic site belongs to panel (a) and that presenting the number of oxygen molecules to panel (b). Only after reading the legend it becomes clear that both of them are parts of panel (b). The ticks on the abscissae of the distributions of the number of water and dioxygen molecules in the catalytic site presented in panel (b) of Figure 2 start at the negative numbers of water/dioxygen molecules. Apparently, these graphs are Gaussian fits. In my opinion, it would be more appropriate to present raw histograms. First, the number of data is limited and, second, the respective lobes of the distributions are right-skewed even by definition, because they cannot extend to negative molecule counts.

Page 7, lines 2-4 of the second paragraph. The sentence "Two main oxygen clusters were detected using *WatFinder*, and they were **district** from the conserved waters (**Fig. 2b**, inset, in yellow)." is unclear. What does the word "district" mean in this context?

The scientific results contained in Mr. Manivarma's dissertation can be summarized as follows:

1. Finding a plausible mechanism of the extraction of a lipid molecule from the membrane by the 15LOX-1/PEBP1 complex.
2. Demonstrating the essential role of the P112 residue in the substrate-acquisition process.
3. Finding a plausible mechanism of oxygen-molecule delivery to the catalytic site and proposing the mechanism of lipid peroxidation.

It should be noted that the findings from theoretical modeling are supported by the experimental data from the collaborating experimental group.

To sum up, in my opinion the results contained in Mr. Thiliban Maivarma's Ph.D. thesis are an important step forward towards the understanding of the mechanism of ferroptosis. These results bring in a substantial load of scientific novelty. The dissertation is a piece of excellent research executed at the professional level. The volume of work done by the Candidate is very impressive. My minor critical remarks do not diminish the scientific value of Mr. Maivarna's dissertation. All specific aims of the Ph.D. project have been accomplished. Most of the dissertation material has already been published in excellent scientific journals. The dissertation undoubtedly meets the requirements set for doctoral theses under the Act of July 20, 2018 – Law on Higher Education and Science (consolidated text: Journal of Laws of 2022, item 574, as amended), as well as the customary standards set for doctoral dissertations in exact and natural sciences. Therefore, I am asking the High Council of the Physical Sciences Discipline of the Faculty of Physics, Astronomy and Applied Informatics of the Nicholaus Copernicus University to admit Mr. Thiliban Manivarma, M.Sc., to further stages of the Ph.D. procedures.

Prof. dr hab. Józef Adam Liwo