



## Report on Agnieszka Stańczak's thesis

Agnieszka Stańczak has submitted a thesis with the title “Theoretical Investigation of the Enzymatic Reactivity of Coupled Binuclear Copper System(s) “Strongly Correlated” with Experiments”. The supervisors have been Prof. Lubomír Rulíšek and Dr. Martin Srnec. The thesis is based on four scientific articles that are published in high-impact international, ChemPhysChem, Chemical Communication, the Proceedings of the National Academy of Sciences of the United States of America and the Journal of the American Chemical Society and a manuscript. The thesis investigates the reaction mechanism of the dicopper enzyme tyrosinase with computational methods (quantum mechanics, QM/MM, molecular dynamics). It is a tight cooperation with Prof. Solomon's group at Stanford University, performing kinetic and spectroscopic characterisation of various intermediates of the enzyme.

The thesis itself is a 65-page summary of this work. It contains an introduction about the enzymes studied and the methods used. After some short notes about the motivation and the aims of the work, it summarises the work done in the four articles and the manuscript. The thesis ends with some conclusions a discussion of the impact of the work and how it can be continued.

The first article is a benchmark of the performance of various quantum-mechanical (QM) methods to treat binuclear  $\text{Cu}_2$  clusters and especially the peroxo- $\text{Cu(II)}_2$  bis- $\mu$ -oxo- $\text{Cu(III)}_2$  equilibrium. It uses high-level CCSD(T) calculations as a reference and test nine different density-functional theory (DFT) methods. Pure DFT functionals seem to give the best results and TPSS/def2-SVP seem to be an economic approach, partly because favourable error cancellation. This article is purely computational.

The second article (a communication) shows that there are hydrogen-bond interactions between the active site of tyrosinase and second-sphere water molecules. Molecular dynamics (MD) simulations and combined QM and molecular mechanics (MM) calculations suggest that the active site contains four water molecules, of which only one is seen in crystal structures and two form hydrogen bonds to the O atoms of the bound peroxide.

The third article provides a full suggestion of the reaction mechanism of tyrosinase. The protonated (neutral) monophenol binds to the active site by displacing the water molecule and forming a hydrogen bond to the peroxide. The peroxide O–O bond is cleaved and the substrate proton is transferred to one of the O atoms. The other O atom hydroxylates the substrate, which then coordinates to Cu.

The fourth article supports the suggested mechanism by providing kinetic and computational evidence from various para-substituted phenols for an initial proton transfer between the protonated phenolic substrate and the Cu<sub>2</sub>O<sub>2</sub> catalytic core.

The manuscript suggests a mechanism of the oxidation of catechols to quinones. First, the *sp*<sup>3</sup> intermediate is converted to a catecholate intermediate by proton transfer involving a water molecule. Then, the catecholate is oxidised and the quinone is released. A reaction barrier was found similar to what is seen experimentally.

The thesis presents a large amount of important and interesting scientific work. The investigations are methodologically and technically sound. The candidate has apparently done most of the computational work. She is the first or a shared first author (for the combined experimental and computational studies) of the articles. The published articles are well written with a clear and convincing logic. The combination of experiments and calculations is a strength and the cooperation is with a world-leading group.

I enjoyed much reading the thesis. It is well written and interesting to read. However, the language can be improved in some aspects, in particular regarding the use of “the” and “a”. It was clearly a bit harder to follow the manuscript than the published articles. Of course, the missing methods section also makes the understanding harder.

In summary, the thesis involves a significant amount of high-level scientific work carried out by the candidate during her PhD studies. It is my pleasure to recommend that it is accepted as material for PhD defence and ultimately for awarding the PhD title.

Naturally, such an interesting thesis gives rise to a number of interesting questions and discussion issues. Below, I list some of the most interesting ones.

1. In several of the suggested mechanisms, molecules bind to or dissociate from the active site. I would like to discuss the reference state of the free molecules and how their entropies can be estimated.
2. How were the DFT methods in the various studies selected, especially in view of the calibration in the first article?
3. Why was the vibrational analysis performed with all atoms frozen, except for the Cu<sub>2</sub>O<sub>2</sub> core? Does that provide accurate frequencies? Likewise, why were the calculated frequency of the B<sub>3u</sub> mode estimated as the weighted average of the frequencies of all vibrational modes with significant B<sub>3u</sub> character (paper II)? Does that provide an accurate description of the Cu–O mode?

4. Is it proper to keep the active site fixed when performing molecular dynamics simulations to judge the solvation of the active site? Could not the dynamics of the active site affect the binding of water?
5. Why are not the active-site water molecules suggested in paper II observed in crystal structures? Are water densities obtained from MD simulations comparable to that observed by crystallography?