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Review of the PhD thesis entitled „Ternary complex formation of His2 peptides in the presence of Cu(II) ions. A quantitative analysis of selected human and yeast peptides", prepared by Karolina Bossak-Ahmad

The PhD thesis was prepared in the Department of Biophysics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw, under supervision of prof. Wojciech Bal and dr Tomasz Frączyk. The subject of the PhD thesis is under studies in this research group for several years, which resulted in many excellent scientific achievements, published in very well-known, renowned journals.

The PhD thesis was prepared in the form of the set of 5 experimental papers (published twice in Inorganic Chemistry, and once in Chemical Communications, ChemBioChem, and International Journal of Molecular Sciences). PhD candidate serves as the first author in 4 papers, and as the third author in one paper, thus confirming her leading role. In addition, short introduction, description of main achievements presented in each paper and general discussion were attached in the Author's review, supported by 213 references.

In the Introduction section, Ms. Karolina Bossak-Ahmad presents the background of studies performed, with short description of the literature data demonstrating current knowledge on copper-dependent proteins and their functions. Particular attention was paid to His2 peptides examined in the PhD thesis, found in yeast pheromone (∝-factor, WHWLQLKPGQPMY), human wound healing factor (GHK), and model peptide with the bis-His motif (AHH), as well as to urocanic acid (UCA), human serum albumin (HSA), and human copper transporter 1 (hCTR1).

Peptides and proteins comprising histidine residues at the second position, numbering from the N terminus, are very common and have been studied so far; however, our knowledge in this field of science is still incomplete. The main aim of the PhD thesis was to describe the formation of ternary complex between Cu(II) and His2 peptides and imidazole bearing ligands in regard to explain their biological role. Particular parts of the PhD thesis were focused on *Saccharomyces cerevisiae* ∝-factor, model AHH peptide, and human wound healing factor. The aims of the PhD thesis are clearly defined and the results obtained fully answer questions

asked in this thesis. Data gained from PhD studies focused on each particular aim, with additional results added by co-authors, were described in the published papers in detail, and shortly presented in the Author's review. Each chapter describing achievements of the PhD candidate is summarized in the form of main conclusions. This is very helpful in understanding of several detailed data included in each paper. Next chapter presents extensive discussion on the data gained in the PhD thesis in relation to the literature data and future perspectives. Very useful is also presentation of the contribution of the PhD candidate in each paper, demonstrating her leading role. This was also confirmed by all co-authors by description of their individual contribution to each paper.

Based on the content presented in the PhD thesis and published papers, it is obvious that Ms. Karolina Bossak-Ahmad gained broad and advanced practical skills in several biophysical methods, mainly those applying UV-visible and CD spectroscopy, EPR, and NMR. Some of these experiments were carried out also during her short visit in the University of Melbourne or under supervision of international collaborators of the group. She was also responsible for planning experiments, analysis and interpretation of data obtained, and active preparation of manuscripts.

Important findings of the PhD thesis, presented in the published papers, comprise:

1) Demonstration that yeast ∝-factor is biologically active in the form complexed with Cu(II), as well as may bind imidazole group present in culture media components or in proteins present on the cell surface; this might influence interaction with receptor, affect cellular signaling, and scale up the effective affinity of copper binding.
2) Detailed structural analysis of chimeric AHH peptide in complex with Cu(II) in regard to biophysical properties allowed to demonstrate natural switch between two binding sites, 3N and 4N (differing in copper coordination mode through 3 or 4 nitrogen atoms); stability of the complex formed may be altered by external stimuli within the second coordination sphere.
3) Examination of interaction between external ligands and the above-mentioned complex showed that ligands may influence Cu(II) complex formation and its stability, shifting the equilibrium of 4N coordination to higher pH values and creating new ternary 3N+1N complex.
4) Demonstration that new binding constant for copper binding to the ATCUN (N-terminal copper- and nickel-binding site, N-terminal site of peptides and proteins bearing His3 residue) in human serum albumin can serve in copper trafficking in blood and allow Cu(II) transfer from human serum albumin to human copper transporter 1.

5) Demonstration that ternary complexes of Cu(GHK)(L)may form significant component of copper reservoir in natural moisturizing factor.

Results obtained are novel and add important knowledge to the general field of copper homeostasis and Cu(II) engagement in a variety of crucial multilayer processes occurring in complex *in vivo* environment. Biophysical approach undertaken in these studies allowed to discover involvement of Cu(II) in selected interactions at molecular level, taking into consideration influence of environment's components; all the peptides examined formed ternary complexes of Cu(GHK) with imidazole donor. Main PhD conclusions drawn by Ms. Karolina Bossak-Ahmad confirmed that completing the coordination sphere of 3N complex with N^{Im} present in external ligands increases the apparent affinity towards Cu(II), thus making such complexes good competitors for the ATCUN motif. The ability of 3N+1N complexes to withdraw copper from traditional 4N complex brings new possibility of Cu(II) shuttling in human plasma and may be responsible for some mechanisms of Cu(GHK) functions.

Although the PhD thesis is complete and the data gained in the PhD thesis were already positively accepted by the journals' reviewers, additional general discussion would be interesting in understanding complexity of copper homeostasis.

1) Human serum albumin binds a variety of ligands at different sites, with varying affinity (*e.g.*, heme, drugs, lipids). How binding of those ligands may influence or be influenced by Cu(II) binding to His2 peptide ?
2) How pathological states resulting in modification of protein structure (*e.g.*, diabetes) may influence proper copper binding to the target peptides ?
3) What could be the biological advantage of ternary complexes formation between Cu(II), His2 peptide and imidazole, other than allowing more efficient copper transport ?

The main role of the reviewer is to evaluate the PhD thesis content. However, it is worth noting that in addition to 5 published papers, composing the PhD thesis, Ms. Karolina Bossak-Ahmad is the co-author of 10 additional papers published in renowned journals (*e.g.*, Angewandte Chemie, Metallomics, Chemistry). Therefore both achievements presented in the PhD thesis and additional results published in those papers one may rate very high. This was also confirmed by positive reception of the journals' reviewers. Therefore, taking into consideration high value of scientific achievements and their publication in renowned journals, I suggest prizing of the PhD thesis.

The reviewer's overall opinion is very enthusiastic. This is very elegant, well prepared, and well written PhD thesis. The overall content and presentation of this thesis is satisfactory. In conclusion, taking into consideration scientific merit of the PhD thesis prepared by Ms.

Karolina Bossak-Ahmad, I state that the PhD thesis meets all requirements for doctoral dissertations stated in the article 13 of the Act from 14th March 2003 on academic degrees and academic title and on art. degrees and art title (Dz. U. z 2003 r. Nr 65, poz. 595; z 2005 r. Nr 164, poz. 1365, z 2010 r. Nr 96, poz. 620, Nr 182, poz. 1228, z 2011 r. Nr 84, poz. 455). Therefore, I propose to the Scientific Council of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw to allow for further steps of the procedure for awarding of PhD degree for Ms. Karolina Bossak-Ahmad.