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**RECENZJA rozprawy doktorskiej**

**mgr Manuel David Peris-Diaz**

*Biofizyczna charakterystyka*

*częściowo wysyconych form metalotioneiny cynkowej*

*oraz ich rola w komórkowym buforowaniu cynku*

*“Biophysical characterization*

*of metallothionein Zn(II)-loaded states*

*and their role in cellular zinc buffering*

One of the 20th-century biology paradigms was the relationship between structure and function. The activity of any macromolecule should therefore be determined by the specific arrangement of a particular set of functional groups, whose mutual spatial orientation is determined by the "structure-forming" components. The first attempt at such a description was the lock-and-key model postulated in 1894 by Emil Fisher, which in 1958 extended to the induced fit model by Daniel Koshland. Structural studies, therefore, should make it possible to understand the fundamental processes of the living world. Their impact on the development of modern science cannot be overestimated, and many breakthroughs have been honored with a

Nobel Prize. A subjective list of them: **1962** Max F. Perutz, John C. Kendrew "for their studies of the structures of globular proteins"; **1962** Francis H. C. Crick, James D. Watson, Maurice H. F. Wilkins "for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material"; **1982** Aaron Klug "for his development of crystallographic electron microscopy and his structural elucidation of biologically important nucleic acid-protein complexes"; **1991** Richard R. Ernst "for his contributions to the development of the methodology of high resolution nuclear magnetic resonance (NMR) spectroscopy"; **2002** Kurt Wüthrich "for the development of methods for identification and structure analyses of biological macromolecules [...] for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution"; **2002** John B. Fenn and Koichi Tanaka "for the development of methods for identification and structure analyses of biological macromolecules [...] for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules"; **2017** Jacques Dubochet, Joachim Frank, Richard Henderson "for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution".

At the moment, modern structural biology goes beyond the structure, and among others, many functional unstructured proteins (IDP; intrinsically disordered protein) have been identified. Moreover, proteins are heterogeneous. Even macromolecules of the same sequence can differ by modification pattern or ligand bound, both of which may affect their activity. Any changes may be static, location-dependent, or dynamic, occurring in response to changes in the (micro)environment. M. Sc. Manuel David Peris-Diaz dealt with such a complicated system – variously Zn-loaded human metallothioneins, including its role in zinc homeostasis.

The evaluated dissertation was performed at the Department of Chemical Biology, Faculty of Biotechnology of the University of Wrocław, under the supervision of Prof. Artur Krężel, Ph.D. The presented work contributes to long-term studies on zinc-binding proteins in Prof. Krężel's group. The starting point was the role of metallothioneins in zinc homeostasis. The dissertation was prepared in the form of a so-called "clip" (*spinka*) based on five manuscripts from 2020-2021. All these papers have been published in prestigious journals (Trends in Analytical Chemistry, Bioelectrochemistry, Analytical Chemistry, Journal of Proteome Research, Journal of the American Chemical Society) with a total impact factor exceeding 45. They have already been cited more than 40 times (36 without self-citation, Web of Science,

20.9.2022). In these publications, the Ph. D. candidate is always the first author, so his contribution must be assessed as significant.

The dissertation is written in English, has a total of 130 pages, and is divided into six main chapters: Abstract (also in Polish), Introduction, Research Aims, Results and Discussion, Summary and Outlook, and Reference List, 244 positions of which mainly refer to publications newer than 15 years. Four page-length Appendix contains a short CV, a list of publications authored by the candidate, and acknowledgments. The original publications were not collected separately but supplemented with short individual comments contributing to the Results and Discussion section.

Regardless of the high scientific content, the reviewer must draw attention to the editorial deficiencies present in the dissertation. The title page appears in two language versions - Polish and English. In his opinion, they are not equivalent. According to the Polish version, the subjects of the study are **partially saturated forms of zinc metallothionein** (“*Biofizyczna charakterystyka częściowo wysyconych form metalotioneiny cynkowej...*”). In contrast, in English, they are **Zn(II)-loaded states of metallothionein** (“*Biophysical characterization of metallothionein Zn(II)-loaded states*”). Therefore, the latter title does not imply that the Ph. D. applicant studied partially saturated forms of the unspecified isoform of metallothionein but states that the protein was loaded with Zn(II), which information was absent in the Polish version.

A similar inconsistency concerns the **Abstract**, the two versions of which differ slightly. Thus, the cellular concentration of free zinc is listed in the English version, while MT binding affinities towards Zn(II) are reported in the Polish one. Both versions claim that the protein must exist partially Zn(II)-loaded. However, such a thesis should be supported by both concentration and affinities. Moreover, the Polish version states that the study focused on human MT. This lack of diligence makes the reviewer a little bit frustrated. However, he must agree that both versions logically present the tasks undertaken, explain their motivations, and announce the results obtained.

The **Introduction**, described as Chapter 1, is the next part of the dissertation. As expected, it presents the current state of knowledge in all aspects of research related to the dissertation. In particular, the zinc metalloproteome, mammalian zinc metallothioneins, structural studies on metallothioneins, biological Mass Spectrometry and its applications in the studies of mammalian MTs, principles of computer simulations, and, finally, applications of

electrochemical methods assisted by chemometrics in studies of MTs are briefly described. The chapter's content documents the applicant's deep knowledge. He provided all the necessary information and simultaneously critically discussed the possibilities and limitations of particular experimental methods, the spectrum of which (Mass Spectrometry, in silico modeling, Electrochemistry) inspires the reviewer's respect. There are, however, a few comments on this part.

1. Page 2, line 5: error in the pattern for Zn binding site. Should be  $\text{CH}_{2-4}\text{CX}_n\text{HX}_{3-4}\text{H}$
2. Page 2: Is the metal binding equivalent to a charge donation?
3. Page 7: The legend of Fig 1 is probably truncated at the end.
4. Page 9, line 5. It should be a reference to Fig 5, not to Fig 1.

The reviewer would also ask the candidate to address the following questions that arose after reading the Introduction.

- Page 3: Could the candidate comment on the estimates of free zinc concentrations in view of Prof. Bal papers in Metallomics (DOI: 10.1039/c3mt00007a) and Inorganic Chemistry (DOI: 10.1021/acs.inorgchem.9b02419)
- Page 9. Could MT  $^{13}\text{C}$  labeled at specific positions help NMR studies of Zn binding?
- Page 15. Could the alkylation of free Cys residue upon preparation of the protein sample affect the binding preferences of the proximal residues? How static is the location of Zn at particular Cys residue? Could undergo a dynamic change of Zn location in the complex with MT?

The **Research Objectives** are listed in Chapter 2.

1. Description of the Zn-binding mechanisms, including cooperativity and reversibility of binding/dissociation phenomena.
2. Development of experimental and in silico methods that enable studies of zinc cluster organization in  $\text{Zn}_{1-7}\text{MT}$  complexes at residue resolution.
3. Identification of physicochemical properties that drive zinc binding at an atomic level.

The Ph.D. student succinctly presented a sequence of three issues, the implementation of which should contribute to the knowledge of the nature and selectivity of Zn binding by MT. Bearing in mind the difficulties in spectroscopic studies of Zn(II)-binding by MT, the reviewer must agree that in this specific case, the development of experimental methods may have proved to

be one of the goals of the Doctoral Thesis. However, the application of *in silico* methods (e.g., steered molecular dynamics and well-tempered metadynamics) should be treated as a tool rather than the aim of the research.

The **Results and Discussion** section is organized in a somewhat non-standard form, presenting publication after publication. A completely new idea is a Brief History, i.e., the Ph. D. candidate's reflection concerning the behind-the-scenes story of the creation of each publication. The reviewer has never encountered such information before, although he must admit that it raises his subjective evaluation of the work. Such a form remains acceptable, but the reader could easily overlook some vital information. For example, the different isoforms of MT were studied by voltammetry and mass spectrometry, but the implications of this were nowhere discussed. Each contributing publication was preceded by a summary, briefly summarizing the results. The reviewer did not assess the quality of the presented data since all of them were subjected to the journal review processes. In the reviewer's opinion, the presented publications constitute a logical sequence of studies that comprehensively describe the analyzed system. Two of them can be considered methodical (Trends in Analytical Chemistry, Analytical Chemistry). Another two present preliminary stages of research (Bioelectrochemistry, Journal of Proteome Research). The most important is the publication in the Journal of the American Chemical Society, which summarizes the research. Combining experimental MS-derived data with various types of *in silico* simulations, the Ph. D. applicant determined the sequence of zinc-binding events. Furthermore, he pointed out the principal differences between zinc and cobalt binding, thus demonstrating why former studies on cobalt binding led to false interpretations concerning partially loaded Zn(II)-MT complexes.

The **Conclusions** section (Chapter 8) summarizes the results obtained. It is the only part of the dissertation that confronts all the results. Thus, it additionally states the equivalent of a guidebook to the set of publications.

The attached **CV** informs about three sources of funding obtained by the doctoral student (Erasmus Scholarship, NCN Prelude, and NCN Etude). Moreover, based on acknowledgments in publications, it could be presumed that the study was also partially funded by two NCN Opus grants led by the Promoter. The reviewer feels that the acknowledgments on page 117 should include such information.

The Scientific Achievements lists 18 publications, five of which were included in the dissertation. However, there is no information about participation in conferences or awards received (which was noted in the CV)

In conclusion, the reviewer expresses his appreciation for the evaluated work. The Ph. D. student successfully dealt with the not easy task of analyzing such a very heterogeneous system. Despite apparent deficiencies at the editorial level, the dissertation achieves the stated objectives. The main drawback seems to be the atomized description of the results in the individual publications, with the lack of a collective description of the main results. It appears, however, that the last of the included publications, "An Integrated Mass Spectrometry and Molecular Dynamics Simulations Approach Reveals the Spatial Organization Impact of Metal-Binding Sites on the Stability of Metal-Depleted Metallothionein-2 Species," presents an almost complete description of the studied system. Finally, it can be concluded that such an organization of the doctoral dissertation could be justified in this particular case.

The reviewer unquestionably concludes that the evaluated dissertation significantly contributes to the knowledge of the function of metallothioneins as Zn(II) sponges involved in zinc homeostasis. It meets all the requirements for doctoral dissertations specified in the Law on Higher Education and Science of 20.07.2018 (ustawa Prawo o szkolnictwie wyższym i nauce z 20.07.2018 r). Given the above, the reviewer recommends the Scientific Council of the Discipline of Biological Sciences of the University of Wrocław to admit M.Sc. Manuel David Peris-Diaz to further stages of the doctoral procedure. At the same time, taking into account the high level of research and its impact on the field expressed, among others, by the number of citations, he suggests nominating this dissertation for the award.