



dr hab. Agnieszka Kiliszek, prof. IBCH
Zakład Badań Strukturalnych RNA
Instytut Chemii Bioorganicznej
Polskiej Akademii Nauk
ul. Noskowskiego 12/14
61-704 Poznań

Review
of the dissertation by M.Sc. Luca Gessa, entitled: „Structural characterization
and conformational changes of Retinol Binding Protein 3”

The doctoral dissertation submitted for review by Mr. M.Sc. Luca Gessa was carried out at the Institute of Physical Chemistry of the Polish Academy of Sciences. The supervisor of the dissertation is Prof. Maciej Wojtkowski, and the auxiliary supervisor is Dr. Humberto Fernandes.

At the outset, I would like to state that I read the PhD thesis with great interest and pleasure. The thesis describes structural studies focused on the RBP3 protein, which plays an important role in maintaining retinal health by binding toxic retinoids. RBP3 may also be involved in the visual cycle of the retinal chromophore. Nevertheless, its function is not yet fully understood, and one of the main reasons is the lack of a high-resolution structure of RBP3 in complex with retinoids, which could help to explain and rationalize the observed functional properties.

Another important aspect of this research is its medical relevance. Numerous mutations in RBP3 have been associated with various diseases. Therefore, the development of non-invasive methods for RBP3 detection could provide a valuable diagnostic tool in ophthalmological practice.

I would like to highlight three major strengths of this PhD thesis:

1. The research was strategically well designed. The candidate was aware that determination of the RBP3 structure might be challenging and therefore implemented several complementary approaches. The protein was obtained from different sources, including recombinant expression in *E. coli* and isolation from two different species. The expression in *E. coli* involved multiple constructs, including GST-tagged variants. Antibodies were also prepared,

potentially for structural stabilization. Furthermore, the use of two complementary structural methods—X-ray crystallography and cryo-EM—was a very appropriate strategy.

2. The cryo-EM analysis was complemented by additional biophysical and bioinformatic methods, such as SAXS, circular dichroism (CD), tryptophan fluorescence quenching, and molecular docking, which allowed for a broader investigation of the functional properties of RBP3.
3. The thesis is written in a clear and concise manner. I particularly appreciate the inclusion of the author's contribution statement, which provides a transparent overview of the techniques used by the candidate and their level of involvement in the presented work.

I have several minor comments:

First, although the structural descriptions are clear, the figures presenting structural features should include more detailed annotations (for example, Figure 1.14 should indicate α -helices and β -sheets). In some cases, additional figures would improve clarity (e.g., the discussion on proline residues on page 69). The description of docking results could also be presented in a more concise manner. Furthermore, the rationale for antibody preparation is not clearly explained in the text.

I have one point for further discussion:

One of the conclusions of this dissertation is that RBP3 expression should be performed in eukaryotic systems such as insect cells, yeast, or mammalian cell lines. Which of these systems would the candidate recommend and why? Could the candidate clarify whether the observed solubility issues arise from the lack of appropriate chaperones or post-translational modifications? Additionally, if one of the proposed systems yields properly folded protein, what types of constructs or mutants would the candidate design to validate the structural findings, particularly regarding binding sites, structural flexibility, and disease-associated mutations?

In summary, although the primary objective of identifying the ligand-binding sites of RBP3 was only partially achieved, the results obtained represent a significant improvement in the resolution of the structural model. This improved model was used for docking studies aimed at identifying putative ligand-binding sites.

Moreover, the PhD candidate has acquired substantial expertise in protein purification from various sources using different techniques, as well as in structural analysis employing cryo-EM, SAXS, and other biophysical methods. He presented his results in a clear and well-structured manner, demonstrating scientific maturity.

The results have been published in *Open Biology*, with Mr. Luca Gessa as a co-first author. In addition, he is a co-author of two other publications.

Overall, the dissertation constitutes a valuable contribution to the field of structural biology and provides a solid foundation for further studies on RBP3 and its functional role.

I consider that the reviewed doctoral dissertation by Luca Gessa fully meets the requirements for doctoral theses as specified in the relevant provisions of the Article 187 of the Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended), and I recommend that the Scientific Council of Institute of Physical Chemistry of Polish Academy of Science admits Luca Gessa to the subsequent stages of the proceedings for the award of the doctoral degree.

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Agnieszka Kuliszek