



Review of the PhD thesis of Luca Gessa, M.Sc.

Entitled: “Structural characterization and conformational changes of Retinol Binding Protein 3”

M.Sc. Luca Gessa has prepared the presented doctoral dissertation under the supervision of Prof. Dr hab. Maciej Wojtkowski and the auxiliary supervision of Dr. Humberto Fernandes, at the Institute of Physical Chemistry, Polish Academy of Sciences (IPC PAS) in Warsaw, within the framework of the International Centre for Translational Eye Research (ICTER).

General overview of the dissertation

The dissertation is a scientific monograph focused on the structural characterization of Retinol Binding Protein 3 (RBP3), also known as Interphotoreceptor Retinoid-Binding Protein (IRBP). RBP3 is a large glycoprotein (~1300 amino acids in mammals) that resides in the interphotoreceptor matrix of the retina and plays a central role in the retinoid visual cycle by transporting retinoids between the photoreceptors and the retinal pigment epithelium (RPE). Despite its established physiological and pathological relevance, the structural biology of RBP3 has remained remarkably incomplete. This thesis directly addresses this knowledge gap through a multi-technique approach combining cryo-electron microscopy (cryoEM), small-angle X-ray scattering (SAXS), molecular docking, and biophysical characterization.

The thesis is well structured, following the classical format of a scientific monograph. It consists of an extensive Introduction (Chapter 1), Materials and Methods (Chapter 2), Results (Chapter 3), Discussion (Chapter 4), Conclusions (Chapter 5), References, and two Appendices. The text is accompanied by a list of abbreviations and abstracts in both English and Polish. The thesis contains approximately 136 pages of main text, two appendices with supplementary data, and a comprehensive reference list of over 200 citations. The results of this thesis have contributed to three publications, including a co-first-author paper in *Open Biology* (Kaushik*, Gessa* et al., 2025), which constitutes the central published output of this work.



Analysis of the thesis content

Introduction

The Introduction provides a comprehensive and well-organized literature review that guides the reader from the general anatomy of the eye and retina to the specific molecular details of the retinoid visual cycle and RBP3. The candidate demonstrates a solid command of the field, covering topics such as the phototransduction cascade, the RPE-photoreceptor interface, and the role of RBP3 in retinal physiology and pathology. The subsections dedicated to RBP3 gene evolution, glycobiology, and disease associations (diabetic retinopathy, retinitis pigmentosa, high myopia) are particularly thorough and place the experimental work in a meaningful translational context. The section on PDE6 as a driver of photoreceptor morphological changes, although somewhat tangential to the main thesis, adds an interesting perspective on the broader retinal physiology. The aims of the thesis are clearly stated, identifying three principal targets: (i) identification of retinoid binding sites at high resolution through crystallography; (ii) structural characterization of full-length RBP3 through cryoEM; and (iii) investigation of ligand-binding-induced conformational changes through SAXS. The Author's contribution section is commendably detailed and transparent, allowing a clear assessment of the candidate's individual role within a collaborative research effort.

Materials and Methods

The Materials and Methods chapter is comprehensive and well structured, covering two parallel experimental strategies: the recombinant production of individual *Bos taurus* RBP3 modules and the isolation and characterization of full-length *Sus scrofa* RBP3 from native sources. The variety of techniques employed is impressive: molecular cloning, multiple chromatographic purification steps (affinity, ion exchange, size exclusion), Western blotting, circular dichroism, fluorescence spectroscopy, cryoEM with single particle analysis, SAXS, molecular docking, and monoclonal antibody production. The descriptions are sufficiently detailed to allow reproducibility and demonstrate that the candidate has acquired a broad technical repertoire during the doctoral training, including an internship at prof. Krzysztof Palczewski's laboratory at UC Irvine.

Results

The Results chapter constitutes the core of the thesis and can be divided into two main blocks. The first block concerns the recombinant production of individual *Bos taurus* RBP3 modules (M1–M4) and tandem module constructs. Despite extensive efforts in cloning, expression optimization (testing



different tags, *E. coli* strains, and conditions), only Module 4 (bRBP3M4) yielded a soluble fraction amenable to purification. While CD spectroscopy confirmed a folded state with mixed α -helix/ β -strand content, the fluorescence enhancement assays demonstrated that the recombinantly produced M4 was unable to bind all-trans retinol. Although these are predominantly negative results, they are scientifically valuable as they highlight the challenges inherent in the heterologous expression of this heavily glycosylated protein and motivate the shift toward the second experimental strategy.

The second block, representing the major achievement of the thesis, focuses on the full-length *Sus scrofa* RBP3. Through an optimized purification protocol from porcine interphotoreceptor matrix wash, the candidate obtained highly pure and homogeneous samples suitable for structural studies. The most significant result is the determination of the cryoEM structure of apo *Sus scrofa* RBP3 at 3.67 Å resolution (EMD-50552, PDB ID: 9FRX). This represents a substantial improvement over the previously reported 7.4 Å structure of *Bos taurus* RBP3 in complex with a monoclonal antibody (Sears et al., 2020). Crucially, the new structure was obtained without the need for an antibody to reduce conformational flexibility, thus providing a more faithful representation of the native conformation of RBP3. The structure confirms the horseshoe-shaped architecture of the four-module protein, reveals the connecting α -helices between modules with characteristic bending at conserved proline/glycine residues, and shows variable local resolution across the molecule, with the central region (Module 2B to Module 4A) being the best resolved.

The molecular docking analysis, performed on the refined structure, identified multiple putative binding sites, including the previously described hydrophobic cleft between domains A and B and the hydrophobic cavity within domain B. Interestingly, the docking analysis indicates stronger binding energies for retinoids compared to fatty acids, which may explain the differential affinity of RBP3 for different ligands. The identification of inter-module clefts as potential transient interaction points is a novel observation.

The SAXS experiments provide complementary evidence for ligand-induced conformational changes. Upon titration with 11-cis retinal and all-trans retinol, RBP3 shows an increase in $D_{\max}$ and R_g , consistent with a modest extension of the protein. Kratky analysis reveals increased flexibility upon retinoid binding, while DHA binding does not elicit the same effect, suggesting that retinoids and fatty acids interact with different regions of the protein. The comparison with earlier negative staining EM



data by Adler et al. (1987) is well discussed, and the argument that SAXS provides a more physiologically relevant assessment of conformational changes in solution is convincing.

Discussion and Conclusions

The Discussion is thorough and well connected to the existing literature. The candidate provides a mature interpretation of both positive and negative results. The evolutionary argument to explain the partial solubility of Module 4 is speculative but intellectually stimulating. The analysis of disease-associated mutations in the context of the new structure (D1080N in retinitis pigmentosa, various truncating and missense mutations associated with high myopia) is one of the most compelling aspects of the thesis, as it demonstrates how the improved structural data can provide mechanistic insights into pathogenesis. The discussion of the “lack of additivity” in ligand binding between individual modules and the full-length protein, and the hypothesis of a conformational gating mechanism, is well articulated, though the candidate correctly acknowledges that a definitive model remains elusive.

The Conclusions are concise and realistic, openly acknowledging the limitations of the work (inability to obtain cryoEM structures of ligand-bound complexes) while outlining a coherent strategy for future investigations, including crystallography and NMR of truncated constructs produced in eukaryotic expression systems.

Remarks and questions for the candidate

The following remarks and questions do not detract from my overall positive assessment of the thesis. I hope they will stimulate a fruitful scientific discussion during the defence.

1. The cryoEM datasets collected for RBP3 in complex with 11-cis retinal, all-trans retinol, and DHA were of insufficient quality for 3D reconstruction. The candidate attributes this to ligand-induced local flexibility causing particle misalignment. Could the candidate elaborate on whether alternative sample preparation strategies — such as cross-linking (e.g., GraFix), the use of nanobodies or Fab fragments, or vitrification under different conditions — might stabilize the ligand-bound complexes? Has the possibility of time-resolved cryoEM approaches been considered to capture transient conformational states?
2. The binding energies reported from the docking analysis (e.g., -2.15 kcal/mol for retinoids in Cleft 1) are relatively modest. At the resolution of 3.67 Å, some uncertainty in side-chain positioning is expected, particularly in regions subjected only to rigid-body refinement. Could



the candidate discuss the confidence level of the docking predictions in terms of the local resolution of the specific binding regions?

3. Regarding Figure 3.9 and the discussion in section 4.1.2, the far-UV CD spectrum (190–260 nm) provides valuable information on the secondary structure content of bRBP3M4; however, the candidate acknowledges that a fraction of the protein may be misfolded. In this context, could the candidate clarify why the near-UV CD range (250–320 nm) — which reports on the chiral environment of aromatic residues (Trp, Tyr, Phe) and is diagnostically informative for the presence of a well-defined tertiary structure, thereby allowing discrimination between a natively folded protein and a molten globule state — was not included in the analysis? Furthermore, extending the CD measurement into the near-UV/visible range (up to ~500 nm) in the presence of retinoids would have enabled detection of any induced CD signal (ICD) arising from ligand binding in a chiral protein environment, providing a direct structural line of evidence for the absence of retinoid binding that would complement and strengthen the interpretation of the fluorescence enhancement assay (Figure 3.10). Were such measurements attempted, and if not, could the candidate comment on whether these experiments are planned or discuss this as an acknowledged limitation of the current structural characterization of bRBP3M4?
4. I would like to draw the candidate's attention to a methodological aspect concerning the fluorescence-based binding assays, which I believe deserves clarification. Two conceptually distinct fluorescence experiments are presented in the thesis, but the distinction between them is not made sufficiently explicit, which may lead to confusion.

The first assay (Figure 3.10, section 2.1.2.6) is a *retinol fluorescence enhancement* experiment performed on the recombinant bRBP3M4: the excitation wavelength is 330 nm (exciting retinol directly), and the emission is collected in the 400–500 nm range. The rationale is that retinol fluorescence increases when it binds within a hydrophobic protein cavity. The second assay (Figure 3.21A–B, section 2.2.7) is a *tryptophan fluorescence quenching* experiment on full-length native RBP3: the excitation is at 280 nm (exciting tryptophan), and emission is collected in the 300–500 nm range. The decrease in the tryptophan emission peak upon retinoid addition is interpreted as evidence of binding. Furthermore, for the all-trans retinol titration, a “modest increase at 480 nm” is reported, which is difficult to appreciate in the figure, while an



inconsistency exists between the emission peak value reported in the Results (~350 nm) and in the Discussion (~338 nm).

My concern regards the interpretation of the tryptophan quenching data. If the proposed mechanism is Förster Resonance Energy Transfer (FRET) from tryptophan to the retinoid — as implied by the reported sensitized emission at 480 nm — then a critical control experiment appears to be missing: the titration of retinoid alone (without protein) excited at 280 nm under identical buffer conditions. Without this control, it is not possible to distinguish between (a) genuine FRET-sensitized retinol emission, which would confirm proximity to tryptophan residues and thus binding within the hydrophobic cavity; (b) direct excitation of retinol at 280 nm, where retinol has non-negligible absorbance; and (c) an inner filter effect, whereby increasing retinoid concentrations compete for excitation photons, producing an apparent quenching that may not reflect a specific binding event.

It is worth noting that the fluorescence enhancement assay of Figure 3.10, which excites retinol directly at 330 nm, is conceptually close to the type of control that would be needed for Figure 3.21. Could the candidate clarify whether a control titration of retinoid without protein (excited at 280 nm) was performed, and discuss which mechanism — FRET, direct excitation, or inner filter effect — most likely accounts for the observed quenching?

5. The SAXS data show that retinoid binding induces conformational extension, while DHA binding does not produce a similar effect (as visible from the Kratky plots). The candidate proposes that retinoids and fatty acids bind to different regions of the protein. Given the available SAXS data and the cryoEM structure, has the candidate attempted to generate SAXS-constrained molecular models (e.g., using CORAL or other multi-domain rigid-body modelling tools) to propose specific conformational states for the apo and ligand-bound forms?
6. The structural analysis of the D1080N mutation associated with autosomal recessive retinitis pigmentosa is well executed. The candidate proposes that the mutation disrupts a salt bridge with R1082 and may lead to misfolding and ER accumulation. Given the expertise available in the laboratory and the purification protocols established during this thesis, would it be feasible to produce the D1080N mutant in a eukaryotic expression system and test its folding, stability,



and secretion directly? Such an experiment could provide direct validation of the structural predictions made in this thesis.

7. The connecting α -helices between modules show a characteristic bend at conserved proline and/or glycine residues, and the candidate suggests this may have a functional significance. Could the candidate speculate on the possible role of these conserved residues in the context of the proposed conformational gating mechanism? Additionally, is there evidence from RBP3 ortholog sequences (e.g., from non-mammalian vertebrates such as zebrafish, where RBP3 retains two modules rather than four) that could shed light on the evolutionary constraints acting on these inter-module junctions?
8. Among the three stated aims of the thesis, the crystallographic identification of retinoid binding sites at high resolution was not achieved, mainly due to the inability to obtain functional recombinant modules. While this is understandable and the negative results are properly discussed, the candidate proposes eukaryotic expression systems as a future strategy. Has the candidate explored or considered insect cell expression (baculovirus) during the thesis work? Given that *Pichia pastoris* and insect cells can provide glycosylation, could the candidate comment on which system might be most suitable and what the expected challenges would be?
9. The monoclonal antibody mAb5 plays a relevant role in this work, both as a tool for validation of the purified RBP3 (Western blot, SEC shift assay) and in the context of the earlier structure by Sears et al. (2020), where it was used to reduce conformational flexibility. However, the specific epitope recognized by mAb5 on RBP3 does not appear to have been mapped. The cross-reactivity between *Bos taurus* and *Sus scrofa* RBP3 suggests that the epitope lies in a conserved region, and the fact that mAb5 works in Western blot may point to a linear rather than conformational epitope. Could the candidate comment on whether the epitope of mAb5 has been characterized in the literature or in unpublished work from Palczewski's group? Additionally, in the Sears et al. structure, the Fab fragment appears to interact near Module 4 — does the candidate have any insight into whether the antibody binding could have influenced the conformation or the ligand-binding properties of RBP3 in that earlier study?
10. A minor editorial remark: the thesis occasionally uses interchangeable terminology (e.g., “binding sites” vs. “transient interaction points” for the shallow clefts identified by docking).



A more systematic and consistent nomenclature throughout the text would enhance clarity. Additionally, some figure legends refer to “data not shown” — in a monographic thesis, it would be beneficial to include such supporting data in the Appendices where possible.

Overall assessment

The doctoral dissertation of Luca Gessa, M.Sc. presents a significant body of original scientific work addressing a relevant and challenging problem in structural biology and retinal biochemistry. The principal achievement — the determination of the cryoEM structure of full-length *Sus scrofa* RBP3 at 3.67 Å resolution without an antibody scaffold — constitutes a genuine advancement over the previously available structural data and opens new avenues for understanding the molecular mechanisms underlying the retinoid visual cycle and RBP3-associated pathologies.

The thesis demonstrates the candidate’s general theoretical knowledge in the discipline, as evidenced by the comprehensive and well-written Introduction, and his ability to conduct scientific research independently, as demonstrated by the breadth of experimental techniques mastered, the critical interpretation of results (including negative ones), and the publication record. The subject matter represents an original solution to a scientific problem, namely the elucidation of the three-dimensional structure of RBP3 at near-atomic resolution and the characterization of its ligand-induced conformational dynamics. The dissertation includes the required abstracts in both English and Polish.

The thesis is well written and well organized. The level of the scientific work is high. I rate the scientific level of the thesis as very good. In my opinion, the doctoral dissertation of Luca Gessa, M.Sc. fully complies with the conditions set out in Art. 187 of the Act of July 20, 2018 – Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended).

In view of the above, I issue a positive review and apply for admission of Luca Gessa, M.Sc. to the next stages of the doctoral dissertation defence.

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