LE STUDIUM
Loire Valley
Institute for Advanced Studies

LE STUDIUM Multidisciplinary Journal

www.lestudium-ias.com

FELLOWSHIP FINAL REPORT

New crystallization strategies to fast-feed structure-guided pharmacological development – Applications to large biological assemblies involved in RNA metabolism

Emmanuel Saridakis[1,2,3], Franck Coste[2], Bertrand Castaing[2], Marc Boudvillain[2]

[1]LE STUDIUM Institute for Advanced Studies, 45000 Orléans, France
[2]Centre de Biophysique Moleculaire, CNRS UPR4301, rue Charles Sadron, 45071 Orleans cedex 2, France
3 Institute of Nanoscience and Nanotechnology, National Centre for Scientific Research “DEMOKRITOS”, 15310 Athens, Greece

REPORT INFO

Fellow: Dr. Emmanuel Saridakis
From Institute of Nanoscience & Nanotechnology, National Centre for Scientific Research “Demokritos”, Athens, Greece
Host laboratory in region Centre-Val de Loire: Aspects Moleculaires du Vivant, Centre de Biophysique Moleculaire, CNRS-Orleans.
Host scientist: Dr. Marc Boudvillain
Period of residence in region Centre-Val de Loire: February 2017-january 2018.

Keywords :
Rho protein, hexameric helicases, RNA metabolism, transcription termination factor, X-ray crystallography, Hofmeister series

ABSTRACT

The elucidation of three-dimensional structures of molecular machines that control cellular physiology is necessary for the understanding of the mechanisms of life and for the development of rational screening tests for pharmaceutical applications. Due to the large size of these biological entities and the high resolution which is sought, X-ray crystallographic structure determination is the method of choice. Obtaining crystals of biological complexes however remains difficult and is the bottleneck to this method. In this project, we have applied sophisticated crystallization strategies to a hitherto intractable problem: crystallising a molecular motor, namely the bacterial transcription termination factor Rho from Mycobacterium tuberculosis. Rho is a ring-shaped hexameric helicase targeting transcriptional complexes and R-loops, and regulating RNA metabolism in a variety of ways. The first crystals of M.tub. Rho have been obtained, which however should now be optimised to reach an X-ray diffraction resolution sufficient for full three-dimensional structure determination. In addition, we have developed a theoretical model describing the varying usefulness of ions at different positions in the Hofmeister series, according to thermodynamic properties of the crystallizing protein.

1- Introduction

Bacterial infectious diseases cause millions of deaths worldwide. In 2012, for example, the World Health Organization estimated new tuberculosis cases to 8.6 million and deaths to 1 million. Furthermore, changes in agricultural practices combined with the increase in human migration have fostered the emergence of novel, hyper-virulent bacterial strains resulting from the horizontal acquisition of virulence determinants and/or antibiotic-resistant genes. A better understanding of the molecular mechanisms that underlie the ability of bacteria to adapt to the environment and express newly acquired genes is quintessential for the development of new therapeutic strategies.

At the heart of bacterial expression programs lies the transcription termination factor Rho, a ring-shaped RNA helicase targeting transcriptional complexes and R-loops. Besides contributing to the punctuation of transcription units in the genome, Rho mediates riboswitch-dependent gene regulation, maintains chromosomal integrity by disrupting transcriptional R-loops and by preventing conflicts between transcription and DNA

replication, and suppresses expression of harmful horizontally-acquired genes [1]. Additional physiological roles and targets of Rho are continuously being discovered [2, 3].

The Rho hexamer is a complex molecular machine requiring the tight coordination of multiple RNA binding sites and six ATP hydrolysis pockets, to achieve the recognition of ~70 nucleotide (nt)-long poorly structured and cytosine-rich *Rut* (Rho utilization) sites within nascent transcripts and subsequent ATP-dependent translocation toward the RNA 3'-end. Due to the great complexity of Rho architecture and despite the wealth of genetic and biochemical data available [1], many central features of its molecular mechanism remain obscure.

Rho is absent from eukaryotes but is essential in many bacteria, thereby being an attractive pharmacological target for the development of new antibiotics [4]. By helping to understand how Rho function is integrated in the bacterial physiology, such tools should ultimately facilitate the development of innovative antibacterial strategies.

A handful of crystal structures of the Rho hexamer from *E. coli* [5-7] and one structure of Rho from *Thermotoga maritima* [8] provide invaluable models of specific Rho molecular state(s) along its physiological reaction pathway but also display features that are not always consistent among the various structures, thereby prompting controversy about the exact mechanisms used by Rho [9-12].

Mycobacterium tuberculosis, like most other gram-positive pathogens, is refractory to the only available anti-Rho drug, bicyclomycin. Interestingly, Rho is expressed and essential for growth in *M. tub*, but appears to operate by a mechanism relying on unusual motor features (e.g. high nucleotide selectivity but low translocation processivity), the structural basis of which is not understood [4, 13]. *M. tub.* Rho contains a large insertion of 144 amino acids in its N-terminal RNA binding domain and non-conservation amino acid substitutions at key catalytic positions of its motor domain that likely contribute to its unusual mechanism of action [13].

Attempts at crystallizing *M. tub.* Rho had proven unsuccessful in the past. Here we report the growth and X-ray crystallographic diffraction data collection from the first crystals of *M.tub.* Rho, the optimization of which may lead to the structure determination of this complex molecular machine.

Part of this Fellowship was spent in developing and applying novel crystallization methodology. Apart from the innovative and sophisticated, but already known techniques used for *M.tub.* Rho crystallization and described in more extensive detail below, the Fellow also developed a theoretical model that relates the differing usefulness of the various ions in the Hofmeister series (i.e. their ranking according to their kosmotropic or chaotropic character) [14], to specific thermodynamic properties of the protein being crystallized.

2- Experimental details

M. tub. Rho was over-expressed in *E.coli* (Rosetta II strains) as described previously [15, 16]. Various constructs were prepared, namely the wild-type, T501K mutant, and two chimeric constructs, consisting of the M.tub N-terminal domain combined with the *E.coli* C-terminal domain and vice-versa.

Purification

Wild-type and T501K proteins were prepared in the context of this project. The published purification protocol [13] was optimized, briefly as follows. The proteins were first purified by cation-exchange chromatography on an SP sepharose FF column (GE-Healthcare) with a 0.15-1 M NaCl gradient, subsequently affinity-selected on a Ni-NTA resin (Thermo Scientific) and finally purified by gel filtration on a Sephacryl S-300 HR column (GE-Healthcare). The proteins were concentrated to ca. 6 mg/mL with a 10000 molecular weight cut-off filter concentrator.

Substrate and ATP-analogue selection

For a stable homohexamer to form in solution, the protein must be bound both to an ATP-analogue and to an RNA- or DNA-oligomer. Extensive stability trials with various combinations of candidate ligands were thus carried out.

To avoid the extremely time- and protein-consuming crystallization screening with all possible ligand combinations, a simple strategy based on Thermal Shift Assay (Differential Scanning Fluorimetry) was adopted. TSA is a quick, rough and inexpensive method to assess which ligands bind to and thermally stabilise purified proteins. Briefly, the protein's unfolding temperature T_m is measured by an increase in the fluorescence of a dye (Sypro Orange) with affinity for the (mostly buried when in the native state) hydrophobic parts of the protein, as temperature is being increased in a thermal cycler [17].

Crystallization

Initial screening with a series of commercially available screens was performed using a Mosquito^{TM} liquid-handling nano-volume crystallization robot (TTP Labtech). 75-200 nL protein stock solution (protein + ATP analogue + oligonucleotide substrate) was mixed with 75-200 nL reservoir solution in sitting drop vapour diffusion mode, at various ratios. Optimization trials were performed using LINBRO-type crystallization plates. They were set up using the vapour diffusion hanging drop method. 1 mL of protein stock was mixed with 1 mL of reservoir solution.

On-line available software XtalPred and CRYSTALP2 was used to compare *M.tub.* Rho's crystallization propensity with that of the more readily crystallizable *E.coli* Rho. It was predicted that increased flexibility of regions of the *M.tub.* protein would result in a much lower crystallization propensity. *In situ* random proteolysis with a series of proteases was therefore tried.

Preliminary X-ray diffraction data collection

The small crystals that were obtained, were cryoprotected and frozen in liquid nitrogen. They were later X-rayed at the SOLEIL synchrotron radiation source, Paris.

3- Results and discussion

ADP.BeF_3 was selected as the most thermostabilising ATP-analogue. In combination with that cofactor, from an extensive series of tested oligonucleotides, two DNA- and two RNA-oligonucleotides had a stabilizing effect on the protein. All the resulting ADP.BeF3/ selected oligonucleotide combinations as well as protein without ligand were brought to crystallization trials.

Initial extensive screening with intact protein did not yield crystals. Limited random proteolysis before incubation of the protein with selected ligands, yielded *M.tub* Rho T501K mutant crystals from two screen conditions that had previously resulted in amorphously precipitating material. These were further optimized (Fig. 1), including with the use of dynamic nucleation-growth uncoupling, a method developed by the Fellow [18, 19].

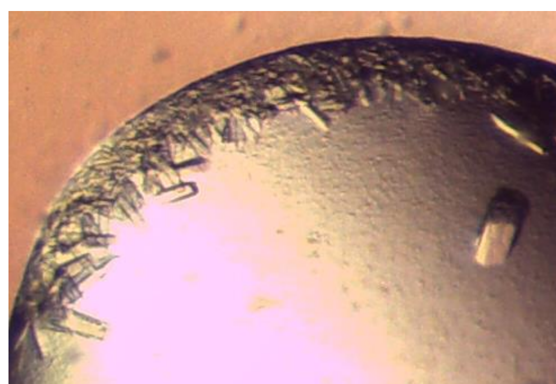

Figure 1. *M.tub. Rho crystals obtained in vapour diffusion hanging drop, after optimization of initial conditions.*

The best crystals diffracted to ca. 5-6 Å. This resolution is insufficient for seeing any structural details, but a molecular replacement solution confirms that, in spite of the limited proteolysis, the crystals consisted of well-defined protein hexamers.

Saridakis, E.; Coste, F.; Castaing, B.; Boudvillain, M. New crystallization strategies to fast-feed structure-guided pharmacological development – Applications to large biological assemblies involved in RNA metabolism, *LE STUDIUM Multidisciplinary Journal*, **2018**, *2*, 1-5
https://doi.org/10.34846/Le-Studium.152.02.FR.01-2018

With respect to the part of the Project that relates to the development of novel crystallization methodology, a correlation was found between some thermodynamic parameters of a given protein and the position on the Hofmeister series of the salts that are best suited to crystallizing it. The protein's thermodynamic stability (Gibbs Free Energy of unfolding) and the hydration contributions to its enthalpy and entropy of unfolding have been seen to determine whether the protein requires a kosmotropic salt as crystallization precipitant (e.g. ammonium sulphate) or if it is equally or more amenable to crystallization from a chaotropic salt (e.g. thiocyanate).

4- Conclusion

An extensive experimental study of *M.tub.* Rho stabilization in the presence of various NTP-analogues and substrate oligonucleotides, resulted in determining which were beneficial and which detrimental to protein stability.

The first ever crystals of *M.tub.* Rho protein were obtained, and confirmed a hexameric ring structure. However, the resolution is not sufficient for visualizing any structural details. Work in progress involves further optimization of crystallization conditions with freshly purified protein, with a view to increasing diffraction resolution to at least 3-3.5 Å. Some parallel work is done in collaboration with other french teams using cryo-electron microscopy.

In terms of crystallization methodology, new physico-chemical insight on the use of Hofmeister salts for crystallization has been gained, with a possible future application to rationalizing the crystallization conditions screening process.

5- Perspectives of future collaborations with the host laboratory

Post-Fellowship collaboration with the former host laboratory has already started. Competent cell material has been sent to the Fellow's home laboratory for purification and crystallization optimization. The Fellow is also advising the cryo-electron microscopy collaborating team on selection of stabilizing co-factors and substrates.

6- Articles published in the framework of the fellowship

One article is under submission:

Emmanuel Saridakis and Marc Boudvillain, *Protein thermodynamic properties, crystallisation, and the Hofmeister series.*

One more article, relevant to the main *M.tub.* Rho study, will need to incorporate additional work (see above) before it reaches publication stage.

7- Acknowledgements

This work was supported by the Le Studium, Loire Valley Institute for Advanced Studies, Orleans & Tours, France under Marie Sklodowska-Curie grand agreement no. 665790, European Commission.

The authors wish to thank Mrs. Annie Schwartz for her precious technical assistance in the laboratory, and Drs. Mildred Delaleau, Cedric Nadiras and Eric Eveno, for useful discussions. E.S. also thanks Dr. Eva Jakab-Toth, director of the CBM at CNRS-Orleans, for introducing him to the AMV group, and the Scientific Committee and the staff of Le STUDIUM and of the CBM, for award of the Fellowship and for general guidance and assistance throughout

8- References

1. Boudvillain M., Figueroa-Bossi N., Bossi L., Curr. Opin. Microbiol. 16 (2013) 118.
2. Bossi L., Schwartz A., Guillemardet B., Boudvillain M., Figueroa-Bossi N., Genes Dev. 26 (2012) 1864.
3. Figueroa-Bossi N., Schwartz A., Guillemardet B., D'Heygere F., Bossi L., Boudvillain M., Genes Dev. 28 (2014) 1239.
4. D'Heygere F., Rabhi M., Boudvillain M. Microbiology 159 (2013) 1423.
5. Skordalakes E., Berger J.M., Cell 114 (2003) 135.
6. Skordalakes E., Berger J.M., Cell 127 (2006) 553.

7. Thomsen N.D., Berger J.M., Cell 139 (2009) 523.
8. Canals A., Uson I., Coll M., J. Mol. Biol. 400 (2010) 16.
9. Boudvillain M., Nollmann M., Margeat E., Transcription 1 (2010) 70.
10. Rabhi M., Gocheva V., Jacquinot F., Lee A., Margeat E., Boudvillain M., J. Mol. Biol. 405 (2011) 497.
11. Schwartz A., Rabhi M., Jacquinot F., Margeat E., Rahmouni A.R., Boudvillain M., Nat. Struct. Mol. Biol. 16 (2009) 1309.
12. Soares E., Schwartz A., Nollmann M., Margeat E., Boudvillain M., Nucleic Acids Res. 42 (2014) 9270.
13. D'Heygere F., Schwartz A., Coste F., Castaing B., Boudvillain M., Methods Mol. Biol. 1259 (2015) 293.
14. Collins K.D., Biophys. Chem. 119 (2006) 271.
15. Kalarickal N.C., Ranjan A., Kalyani B.S., Wal M., Sen R., J. Mol.Biol. 395 (2010) 966.
16. Boudvillain M., Walmacq C., Schwartz A., Jacquinot F., Methods Mol. Biol. 587 (2010) 137.
17. Wernimont A., Edwards A., PLoS ONE 4 (2009) e5094.
18. Saridakis E.E.G., Shaw Stewart P.D., Lloyd L.F., Blow D.M., Acta Cryst D50 (1994) 293.
19. Saridakis E., Chayen N.E., Biophys. J. 84 (2003) 1218.