Characterization of the multiples structural states of the multidrug transporter LmrP by conformational restriction

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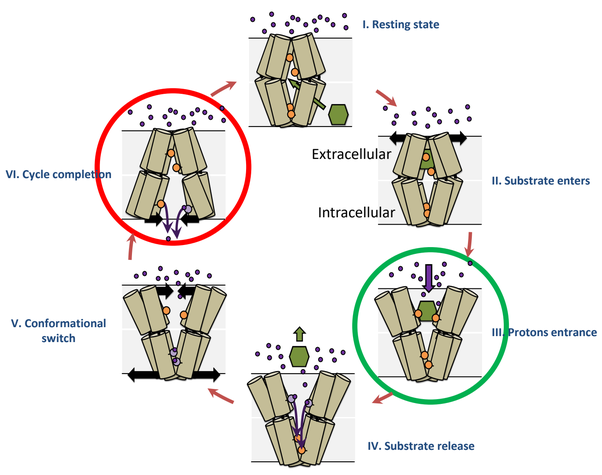
Introduction

LmrP from *L. lactis*, a secondary transporter with remarkable diversity in substrate

We are interested in understanding the molecular basis of multidrug transport. By combining functional and biophysical approaches we intend to characterize structure and dynamics of these transporters. Over the last year we have focused our efforts on LmrP from *L.lactis*, an exporter with remarkable diversity in substrate and extensive functional characterization. Several lines of evidence demonstrate that LmrP must visit different conformations to extrude its ligands. Our goal is to characterize the key conformations at high resolution in order to decipher the molecular basis of transport.

Our laboratory have solved the structure of LmrP in presence of a substrate by X-ray crystallography at 2.9 Å resolution. In those conditions, the protein was stabilized in the outward-open state.



Conformational restriction, how to stabilize different state of LmrP

Different methods are used to stabilize a conformation (Fig1). The first method is **to mutate** specific residues that are involved in **structural stability** of the outward open state. Since LmrP is a secondary transporter, the **pH** will have a huge **structural effect** and as for all transporters, a **ligand** might also **stabilize specific conformations**. A final option is the use of **nanobodies**, which in previous studies have been demonstrated to **promote protein crystallisation** and provide **conformational restriction.**

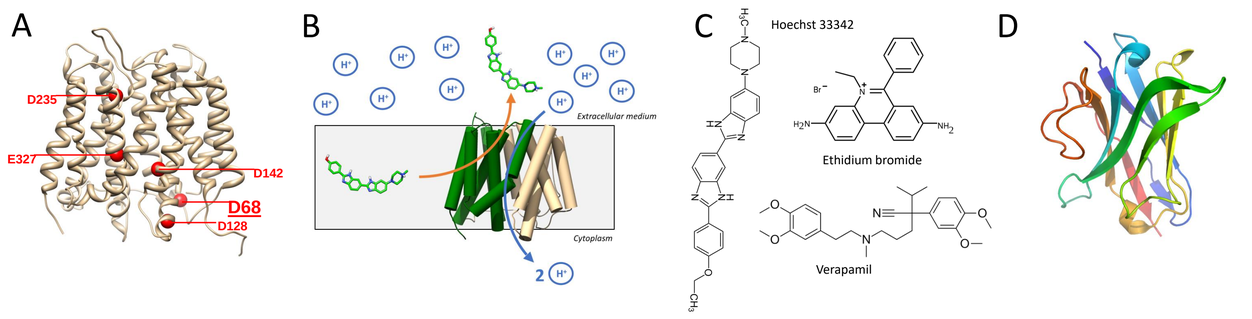


Figure 1: A. Interesting amino acids that can be mutated for conformational restriction **B.** LmrP transport is regulated by a proton gradient **C.** Hoechst 33342 Ethidium bromide and Verapamil, 3 ligands of LmrP **D.** General secondary structure of a nanobody.

Our goal is to obtain structural information on other states of LmrP. We will use two approaches:

- ***X-ray crystallography*, crystals of LmrP with nanobody, ligands and/or mutation could lead to inward open conformation structure.**
- ***Cryo-EM*, is an alternative to X-ray crystallography for membrane protein structure determination of all conformations without the need for crystallization.**

Methods and results

Crystallography

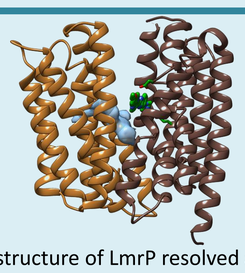


Figure 2: structure of LmrP resolved at 2.9 Å.

- We have solved **structure** of LmrP by crystallography in **outward open** substrate bound state at **2.9 Å** (Fig2).
- **Crystals of LmrP** obtained with **nanobody** and **ligand** of ± **5 Å** resolution (Fig6).

Cryo-Electron Microscopy

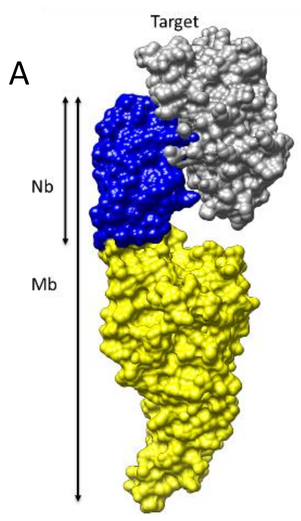
Challenges in Cryo-EM are still huge for small protein. The size **limitation** to have a good signal to noise ratio is around **100 kDa**.

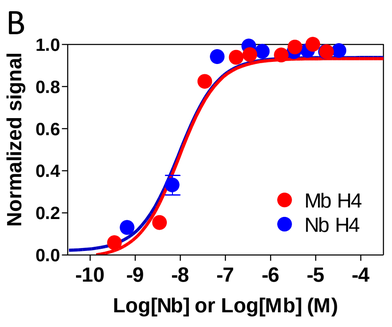
To **solve this problem** we needed a tool that can **increase the size** of LmrP while **keeping the native structure** and **shift the conformation** toward the **inward-open** state.

From nanobody to megabody

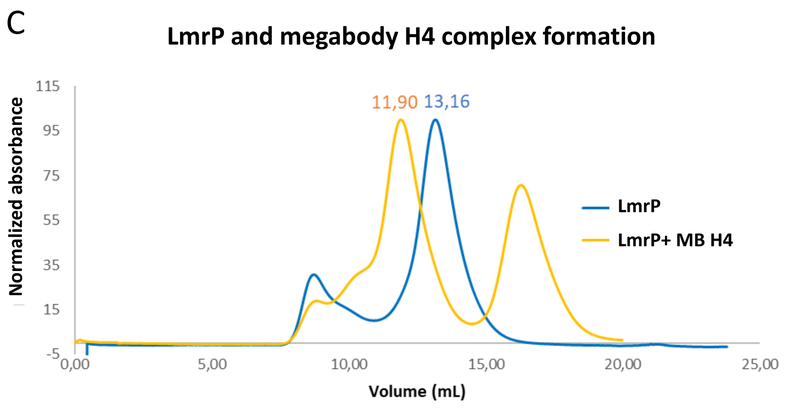
By rigidly fusing a nanobody on a larger protein scaffold, the laboratory of Steyaert, has developed a novel technology, termed **megabodies**, that could be used to increase the size of LmrP particles (Fig3A).

As **nanobodies** recognize a single conformational epitope, the use of **megabodies** offers a direct strategy to **increase the mass of the target** and provide **strong asymmetry** due to the shape of the megabody, which would be of great use during **particle orientation and classification**.



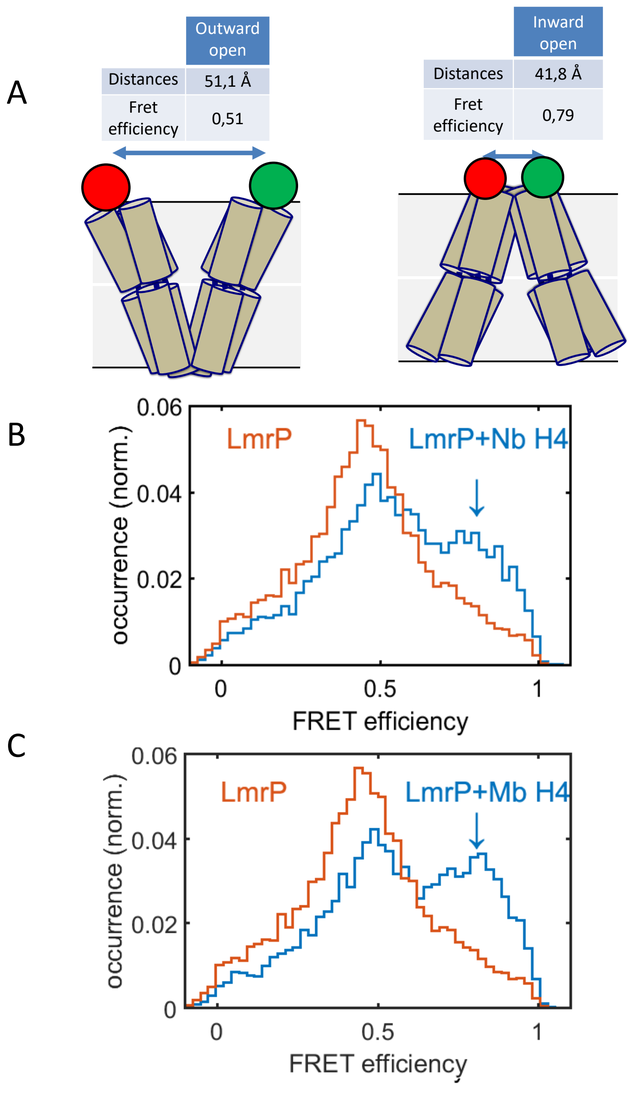


Based on previously isolated LmrP-specific nanobodies, we have obtained a first set of LmrP-specific megabodies and have **confirmed specific binding** by ELISA (Fig3B). Size-exclusion chromatography **confirms the formation of a stable complex** (Fig3C).



Figures 3 A. Overall structure of a megabody. The nanobody (blue) has been raised against its target (grey). It is subsequently genetically fused to the rigid structure of an accessory protein (yellow), forming the extended structure of the megabody. **B**. Elisa assay performed on immobilized LmrP demonstrating high-affinity binding of both Nb H4 and its corresponding Mb. **C.** Size exclusion chromatography of LmrP alone or in complex with the megabody H4.

Conformational restriction



Figures 4 : Nanobody (Nb) H4 and megabody (Mb) H4 stabilize the inward-open state of LmrP. **A.** Donor and acceptor fluorophores are attached to engineered cysteines at positions 164 & 312 (extracellular side) **B.** Single molecule FRET measurements demonstrate that LmrP rests in outward open state in detergent, but the addition of Nb H4 yields a new population (blue arrow) corresponding to the inward open state. The population at 0.5 is still observed most likely due to partial occupancy. **C.** A similar behaviour is observed when adding Mb H4.

We are attempting to stabilize other conformations of LmrP using nano/megabodies. Single Molecule FRET experiments have identified **mega and nanobodies** that **stabilize the inward-open state**. We propose to use such conformational stabilizers in high resolution cryo-EM and crystallography to resolve the missing states.

Preliminary data

Initial negative stain EM of LmrP-Mb complexes confirms the presence of **individual particles of about 10nm**, which agrees with the expected size of the protein complex in detergent micelles (Fig5).

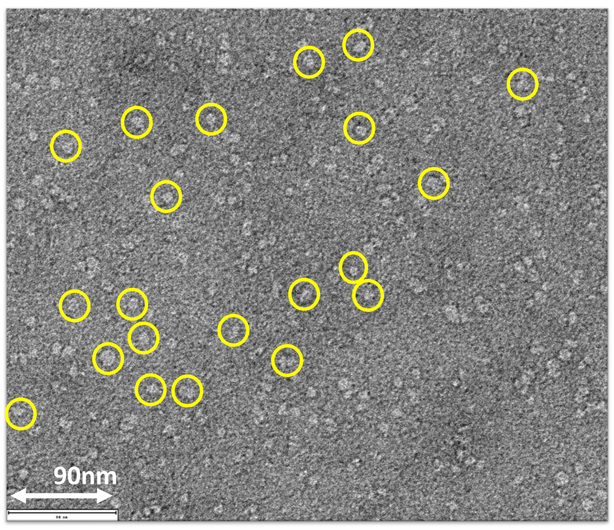
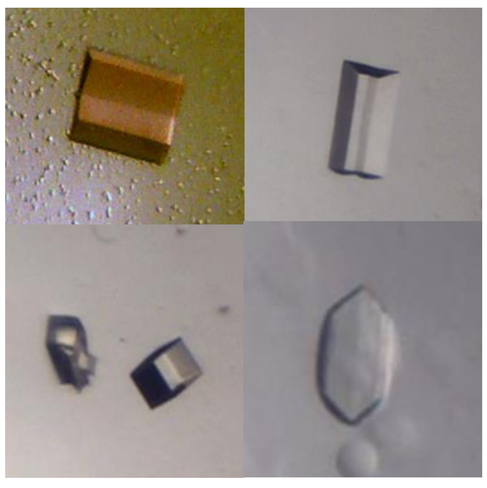


Figure 5 : Negative stain EM image of Lmrp-MbH4 complexes. Shows mostly homogenous particles of ~10-12 nm in size (circled in yellow)



In crystallography, a lot of **crystals have been obtained** with nanobodies. Our best results were :

- **4 Å** diffraction with LmrP + nbH4 + Hoechst33342

Outward open

- **5 Å** diffraction with LmrP + nbH4 + ETBr

Conformation unknown

Figure 6 : Crystals obtained with LmrP and the nanobody H4.
Top left : LmrP + nbH4 + ETBr
Top right : LmrP + nbH4 + Hoechst33342
Bottom left : LmrP + nbH4 + Hoechst 33342
Bottom right LmrP + nbA10 + Hoechst 33342
Size : 100 microns

Conclusion and discussion

We have found conformational stabilizers of LmrP. We will continue to use the nanobody or megabody H4 in crystallography to improve pregressively the resolution of crystals. And for the Cryo-EM, negative stain results are promising and futher optimisations could lead to visible complexes and exploitable images.

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