FP7-MC-IEF

Structural study of a potassium channel, KirBac3.1, using electron microscopy

FINAL REPORT: SUMMARY

Ion channels are membrane proteins that allow the passage of ions across the barrier of the cell constituted by the membrane. As regards the movement through the membrane, three types of proteins have evolved: ion channels, i.e. pores with a selective gating system allowing the passive flow of ions according to their gradients; ion pumps, that transport ions against their gradients using ATP as their energy source; and transporters, that use the spontaneous movement of an ionic species according to its gradient to gain the active transport of another ionic species against its gradient (Ashcroft F.M., Nature, 2006. 440: p.440-7).

Ion channels have crucial roles in many important physiological processes, such as nerve and muscle excitation, blood pressure regulation and cell death. In the human genome, over 340 genes encode for channels, differing for selectivity and tissue localization (Ashcroft F.M., "Ion channels and diseases", 2000, Academic press, New-York). Because of the significant roles played by ion channels, defects in their function, often due to genetic mutations, cause serious diseases, such as diabetes, various types of myotonia and cardiomyopathy, epilepsy and paralysis. The study of structures and functions of ion channels may elucidate their working mechanisms and allow the drug design for various diseases involving transport of ions through the membrane. Moreover, their position on the cell surface, their heterogeneity and the localized expression of some channel types in particular tissues make them an attractive target for many drug therapies. Most notably, anaesthetics, sedatives, antidiabetic and antiviral drugs interacting with specific ion channels have been already developed.

The goal of the present project was the structural study of a bacterial potassium channel, namely KirBac3.1. This channel belongs to the family of inwardly rectifying potassium (Kir) channels, whose function is to regulate membrane potential and K+ transport. The opening and closing (gating) of Kir channels is modulated by numerous intracellular ligands that bind to the channel itself. These include nucleotides (ATP), lipids (PIP2), G-proteins and ions (e.g. H+, Mg2+, Ca2+). In addition, intracellular ions such as Mg2+ and polyamines produce a voltage-dependent block of the pore resulting in inward rectification. Mutations in Kir channel genes cause Anderson's syndrome (Kir2.1), Bartter's syndrome (Kir1.1) and insulin secretory disorders (Kir6.2). KirBac3.1 is a bacterial homologue of the mammalian channels and its structure in the closed state was previously known by X-ray crystallography (Clarke, O. B. et al., Cell, 2010. 141:1018). However, a precise knowledge of the structure in the open state was required in order to elucidate the mechanisms of gating and, thus, open the way to exploit these channels as drug targets.

At the University of Oxford, we studied the open state of KirBac3.1 by means of electron crystallography and X-ray diffraction. The latter technique allows obtaining the structure of proteins from 3D crystals, usually grown in a detergent-containing solution. The advantage of the former technique is that the protein is imaged in a native-like environment, i.e. the lipidic bilayer. However, 2D crystals suitable for electron crystallography experiments are obtained by a careful and mostly manual work of optimization. 2D crystals of KirBac3.1 wild type had been previously obtained in the lab of Dr. Vénien-Bryan in Oxford (Kuo A. et al., Structure, 2005. 13:1463-72). However, the analysis of electron microscopy (EM) images of these crystals revealed a large variability of the unit cell parameters that hampered the 3D reconstruction of the molecular model due to the difficulty of merging different images. Thus, for our study we decided to use mutants of the protein, that were selected to stabilize the open conformation (Paynter, J. J. et al., J. Biol. Chem., 2010. 285(52):40754-61). Our aim was to increase the homogeneity of the starting sample, leading to more regular lattices in the 2D crystals.

The first task of this project was the optimization of the crystallization conditions previously reported (Kuo A. et al., Structure, 2005. 13:1463-72), in order to obtain specimens suitable for the EM data collection at high resolution. For a membrane protein as KirBac3.1, purification in detergent solution is required before crystallization. Starting from the pure detergent-solubilized protein, 2D crystals can be obtained by addition

of lipids, followed by detergent removal. If this protocol is carefully applied and the experimental conditions are suitable, it is possible to observe the formation of a lipidic bilayer, enriched by a high concentration of protein inserted in the membrane-like bilayer. In optimal conditions, the protein forms close packing in the bilayer, thus yielding 2D crystals. In our study, various methodologies were applied to obtain 2D crystals, as predicted in the initial research plan. A thorough systematic screening was conducted testing various crystallization parameters and their influence in the size and quality of the crystals obtained. Besides the original plan, however, we tested a novel methodology, i.e. the cyclodextrin method (Iacovache I. et al., J. Struct. Biol., 2010. 169(3):370-8), for which an automatic system was available, thanks to our collaboration with the group of Professor Henning Stahlberg in the Biozentrum of the University of Basel. This additional tool allowed us to obtain large and well-ordered crystals of a KirBac3.1 mutant. The advantage of these crystals, besides the order of the crystalline lattice that yields high-resolution details in the structural determination, is that the unit cell parameters are less variable than in previous experiments. Therefore, it was possible to merge data from the different images.

According to the initial research plan, once the crystallization conditions were optimized, the following step was to take high-resolution images to produce a 3D reconstruction of the protein in the lipidic bilayer. However, at this point we encountered a problem that delayed the experimental work. The crystals of KirBac3.1 mutants we produced displayed a strong tendency to form stacks. The overlap of different 2D crystals prevents the analysis of data from tilted images, required in order to perform the reconstruction by electron crystallography.

As a side project, not previously foreseen in the research proposal, we undertook 3D crystallization experiments on the same mutants of KirBac3.1 used for the 2D crystallization experiments. The set up of these experiments was possible thanks to the good yield of protein obtained from optimization of the expression and purification protocols. X-ray diffraction data collections were performed on the beamlines of the synchrotron facility of Diamond (Didcot, Oxfordshire, UK). The solved and refined structure showed that the conformation of the channel in the crystals is open. In particular, compared with the previous crystal structures of KirBac3.1, it is possible to observe that the bulky residues that block the ion pathway in the closed conformations are removed from the pore of the channel, thus allowing the passage of potassium ions required by the biological function of this protein (Figure 1 shows a comparison between the pore-lining surfaces of the closed and open states). However, the mutation we introduced in order to stabilize the open conformation introduces a new narrowing point in the ion pathway (not shown in Figure 1). Thus, although the overall conformation of the protein is open, the channel in this crystal structure is not conductive.

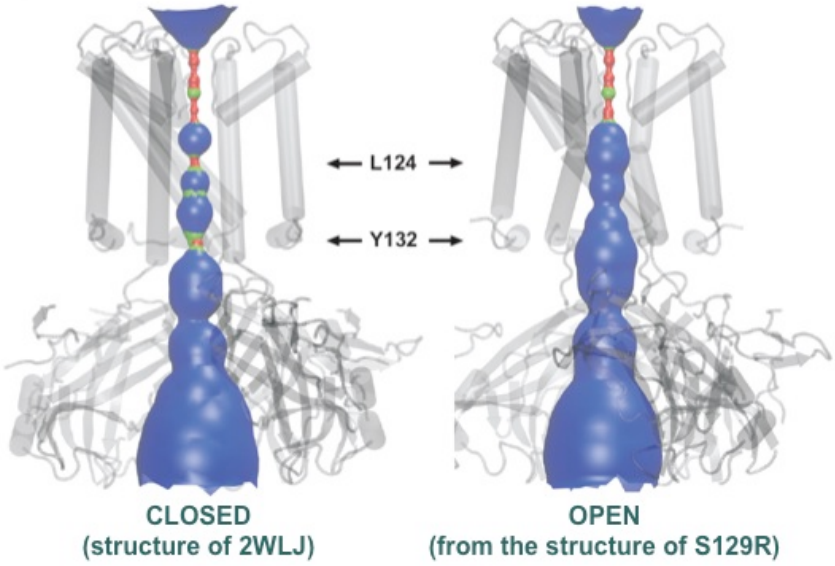

Figure 1: Comparison between the pore-lining surfaces of the closed and open states. In the model of the open state, the mutation has been replaced with the original residue.

The preliminary data from electron crystallography on stacked crystals showed a further opening of the channel in the lipidic environment. We believe the conformational differences we observed to be due to the native-like environment used for the 2D crystals, with respect to the detergent environment of the 3D crystals. This hypothesis is supported by observations made by different research groups on other membrane proteins, as well as by the available data we have from electron microscopy. Thus, a new approach was employed to overcome the formation of stacks of 2D crystals. A careful analysis of the 3D structure suggested that the stacking is driven by strong interactions between the purification tags of protein molecules belonging to different layers. The following experiments to obtain monolayer crystals by cleavage of the interacting residues were successful and the images obtained allowed to obtain a projection map in a direction parallel to the lipidic bilayer. The details of this map (Figure 2), at 6 Å resolution, confirm the hypothesis that a different and wider opening is possible for KirBac3.1 channel when embedded in a lipidic environment. However, the tilted images from these crystals have not yet been collected and, thus, the 3D reconstruction is not available.

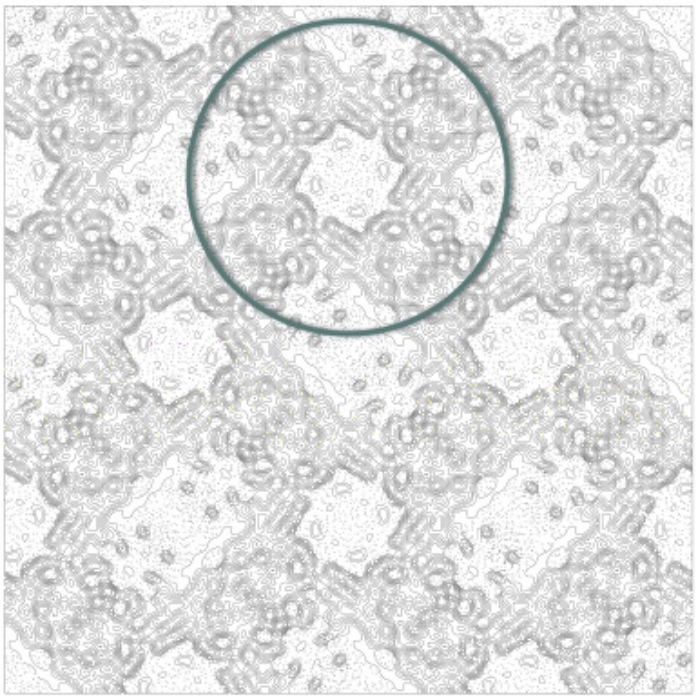

Figure 2: Projection map at 6 Å resolution of the crystals of KirBac3.1 (mutant S129R/Thrombin), obtained by cryo electron microscopy. The circle underlines a single channel.

The work done so far on KirBac3.1 yielded three main results: (i) the determination of the crystal structure of this potassium channel in a open conformation, that was long awaited since it provides new insights into the mechanism of its gating (Figure 3 shows the proposed mechanism of gating of this family of channels); (ii) the thorough screening conducted in order to optimize the 2D crystallization parameters is valuable on a methodological point of view, since it provides useful information for the field of 2D crystallization; (iii) the success in obtaining the monolayer 2D crystals of KirBac3.1 opens the way to the structural determination in a lipidic environment. Therefore, on a biological point of view, the outcome of this research project will be useful for the understanding of mechanisms of gating of the potassium channels belonging to the Kir family, leading in the future to a possible use of these proteins as drug targets. The numerous diseases involving mutations of Kir proteins as well as their presence in many cell tissues make them interesting candidates for pharmacological therapies. On the methodological point of view, our research proposes new perspectives in the 2D crystallization field, underlining the key parameters to obtain crystals and those that have a role in optimization of their order and size. We believe that this information will be useful in setting up 2D crystallization experiments for other membrane proteins.

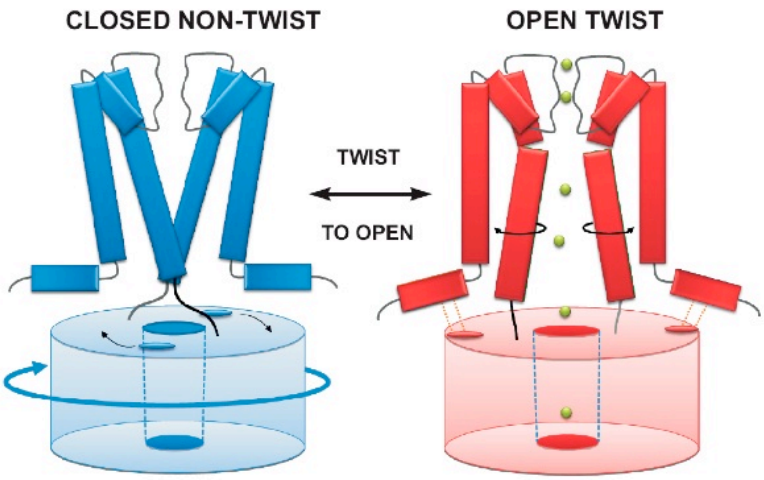

Figure 3: Schematic representation of the proposed mechanism of gating of the Kir channels, based on the structure of KirBac3.1 in an open conformation.