Dynamics of Cyclic Nucleotide-Gated Channels by High-Speed Atomic Force Microscopy

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Ion channels are membrane proteins facilitating ion fluxes across cell membranes, thereby regulating signal pathways for various physiological processes. The activity of ion channels can be triggered by the interaction with secondary messenger molecules and transmit this information into an electrical response by permeation of ions. Important members of this family are cyclic-nucleotide (cN) modulated channels, including nucleotide-gated (CNG) channels, and hyperpolarization-activated and nucleotide-gated (HNC) channels, which are activated by the interaction with cAMP and cGMP. They play a crucial role in the signal transduction of the sensory perception and signaling throughout the whole nervous system. A detailed picture of the specific CNG and HCN structure, their ion transport and a complete understanding of the mechanics of cN modulated ion channel activation are missing. As previously shown, HS-AFM enables to acquire movies at rates of about 10 frames per second with about 1nm resolution, and thus allows the visualization of single molecules at work. In this COST-STSM the first requirements for HS-AFM experiments on cN modulated channels were performed. For this, the optimal sample preparation of MloK1 (prokaryotic homolog of HNC channels) 2D crystals adsorbed on AFM supports was debugged using conventional AFM imaging. Furthermore, first high-resolution images of MloK1 channels in presence and absence of cyclic nucleotides in solution were observed, yielding in the associated structural changes of the ion channel.

Introduction

Ion channels are integral membrane proteins providing the permeation of ions through cell membranes, thereby playing a fundamental role in maintaining various physiological functions. The gating mechanism of these ion channels can be triggered by secondary messenger molecules (*i.e.* ligand-gated ion channels), thereby changing their ion conductance upon ligand binding. The cyclic nucleotide (cN) modulated channels, including nucleotide-gated channels known as CNG channels, and hyperpolarization-activated and nucleotide-gated channels, the so called HNC channels (*a.k.a.* pacemaker channels) belongs to the family of such ligand activated channels. CNG channels recognize the intracellular level of cAMP and cGMP by specific binding of these molecules and transmit this signal in an electrical response by adjusting the flux of ions through the pore channel. The main task of CNG channels is the detection of photons and odorants, and thus they are the main signal transducer in the olfactory and visual sensory system [1, 2]. In contrast, HNC channels are opened for K^+ ions by hyperpolarization and their activity is elevated by increased levels of cN in heart- and brain cells, thereby contributing in pacemaker activity and motor-coordination processes [3, 4]. Both types of cN-modulated channels belong to the S4 superfamily of voltage gated cation channels [5, 6]. Hence, they contain both, a putative voltage sensor and a ligand binding domain. They form tetramers where each subunit consists of six transmembrane sequences (T1-T6, Figure 1). The pore itself is formed by a sequence between T5 and T6, which contains a selective filter, allowing discrimination between certain types of ions. Transmembrane sequences T1-T4 are thought to move in the transmembrane gradient and modify the gating behavior of the channel (*a.k.a.* voltage sensor). Importantly, they have a cyclic nucleotide binding domain (CNBD) at the cytoplasmic COOH terminus which is connected by a 60-80 amino acids long linker to the transmembrane core.

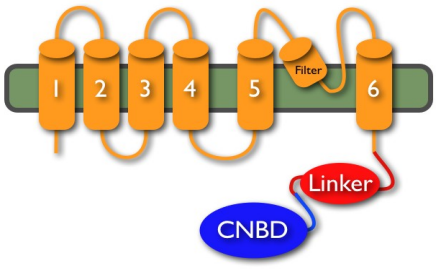


Figure 1: *Scheme of cyclic nucleotide-gated ion channels. CNG as well as HNC channels consist of six transmembrane helixes (yellow). Helices 1 to 4 form the voltage sensor, the 5th and 6th transmembrane domain form the pore channel including the selective filter for potassium ions. The cyclic nucleotide binding domain (blue) is located at the COOH-terminus, which is tethered to the core by a linker sequence (red).*

Recording of the ion current of cN-modulated channels provided valuable information regarding ligand sensitivity, the mechanism(s) underlying channel activation and ion selectivity [7-10]. Recent high-resolution structures of several K+ channels [11-13] with similar topology to CNG and HCN channels yielded models helping in describing the molecular architecture. However, the specific structural details of the cN-binding subfamily, their assembly under native conditions and the sequence of conformational re-arrangements that start with binding cN and finish with the opening of the channel gate are still largely unknown.

CN modulated channels have been extensively studied with electrophysiological techniques and more recently with structural approaches [10, 14-19]. It has been proposed that the binding of cAMP/cGMP causes a conformational change in the CNBD that is translated to and amplified by the linker region that finally opens the channel [1, 20]. Thus, the linker domain must also play a key role in the assembly of HCN channels, as crystal-structures of a soluble construct including both a CNBD and the linker domain from several HCN channels revealed a four-fold symmetric ring structure induced by the interaction between the linker domains [21-23]. Several studies have also proposed a dimer-of-dimers symmetry arrangement within the full-length channels [7, 10, 16].

Thus, so far a detailed picture of the specific CNG and HCN structure, their ion transport and a complete understanding of the mechanics of cN-modulated ion channel activation are missing and under debate. The elucidation of the structure-function relationship on a molecular basis of liganded and un-liganded cN modulated channels allows addressing these questions, discriminate contradictory views and extend our knowledge of these cellular nano-machines.

In this study we used the prokaryotic cN-modulated K+ channel MloK1 from the bacterium *Mesorhizobium loti* due to relatively straightforward expression and purification procedures of prokaryotic proteins compared to their eukaryotic homologos. MloK1 has a high similarity to CNG and HCN channels, although it is lacking the linker domain. As previously shown by electron microscopy and AFM studies [14, 24] MloK1 is a perfect candidate for both structural and functional studies for cN-mediated ion channels.

Results and Discussion

The first step in studying the structural dynamics of MloK1 channels upon cAMP binding to the CNB domain was the optimization of the sample preparation of MloK1 2D crystals on AFM supports. For this, different MloK1 samples were adsorbed on freshly cleaved mica sheets in presence of $MgCl_2$ for about 30 minutes. After rinsing with imaging buffer (10 mM Tris, 150 mM KCl, pH 7.5) AFM imaging in contact mode was applied to characterize to particular specimen. Some samples did not show any MloK1 membranes and only showed bare lipid layers (Figure 2). In contrast, the recently purified MloK1 samples resulted in MloK1 protein 2D crystals. Figure 3 shows an overview AFM image of a protein patch of several micrometers. In the higher magnification images (Figure 4A and 4B) the MloK1 crystal structure could be clearly observed in absence of cAMP. Hence, the molecules are in closed conformation and appear as bulky dots protruding 2.82 ± 0.82 nm from the membrane.

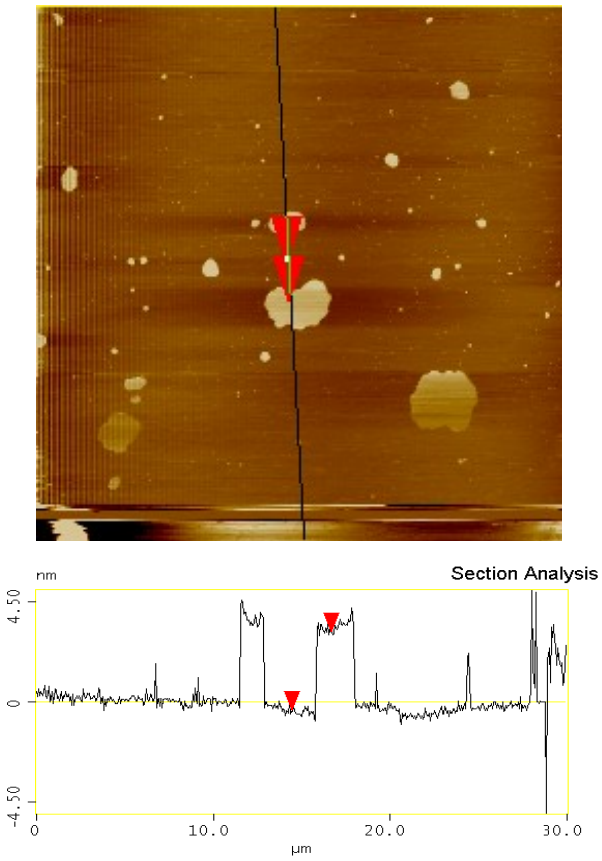


Figure 2: *AFM image of a sample showing only bare lipid batches. Cross-section analyses resulted in a layer heigh of about 3 nm. Scan range of the image is 30 µm and z-scale was adjusted to 10 nm.*

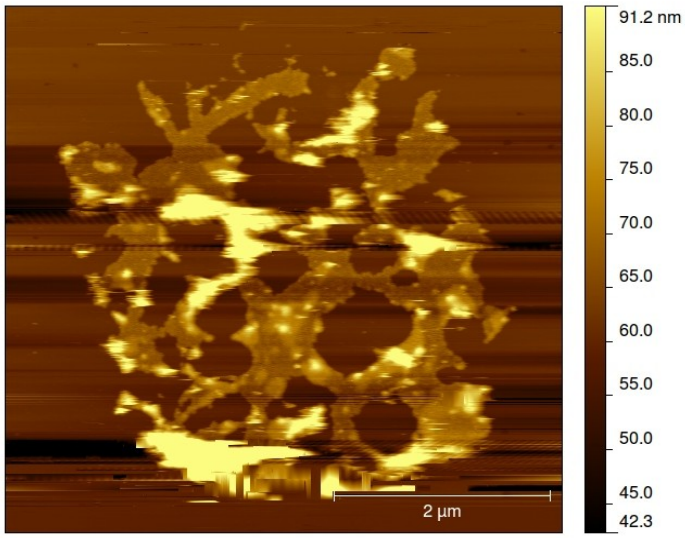
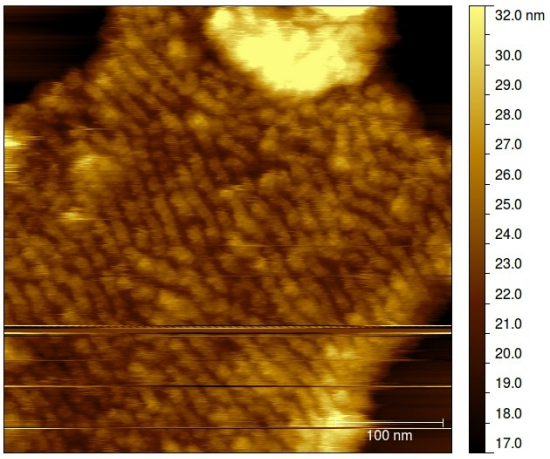


Figure 3: *AFM image of a MloK1 protein crystal without cAMP in solution.*

A



B

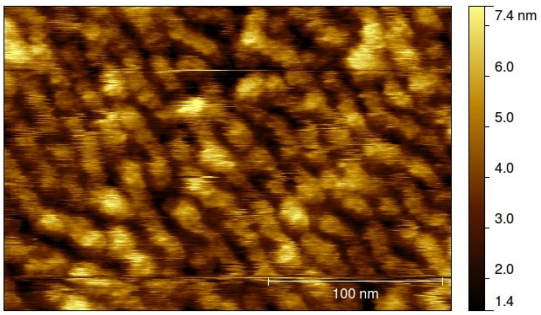


Figure 4: *High magnification views of a MloK1 2D crystal without cAMP in solution.* ***A)*** *Densely packed MloK1 molecules are regularly aligned in the membrane.* ***B)*** *High resolution topographs resolves the single tetrameric molecules which are visible as individual protrusions.*

Notably, the sample preparation tended to form huge aggregates on the mica surface, and thus observing a proper location for high resolution imaging turned out to be quite time consuming. To overcome this problem the sample was adsorbed on mica without $MgCl_2$ but in the presence of EDTA (10 mM). The incubation with EDTA in solution resulted in nearly total extermination of aggregates and in very flat MloK1 membranes (Figure 5).

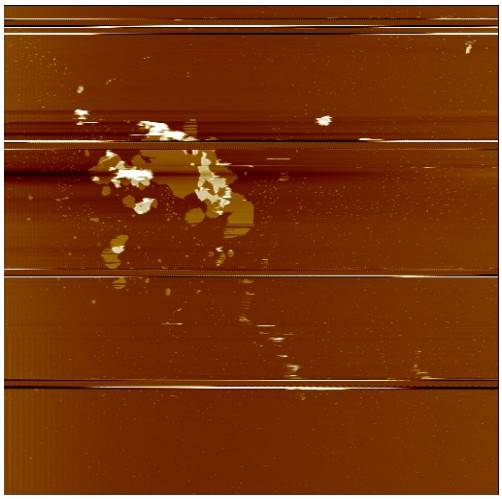


Figure 5: *Overview scan of about 9 µm of MloK1 crystals absorbed on mica in the presence of 10 mM EDTA. (25 nm z-scale.)*

Using this optimized sample preparation the first structural studies were performed. The schematic demonstration of the experimental set-up is shown in Figure 6. MloK1 proteins with bound cAMP were adsorbed on freshly cleaved mica and imaged in high-resolution buffer containing 30 µM cAMP. After acquiring molecular resolution images the solution was exchanged to imaging buffer without ligand and the sample was imaged subsequently at the very same position again.

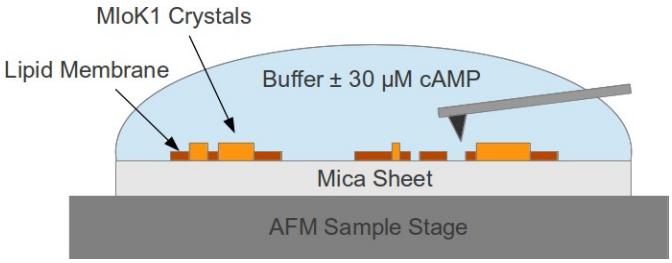


Figure 6: *Illustration of the experimental set-up. MloK1 crystals were immobilized on freshly cleaved mica sheets and imaged in contact mode, AFM in imaging buffer with and without cAMP in solution.*

The following Figure 7 A and B shows high resolution images of a MloK1 membrane with bound cAMP to the CNB domain. The tetrameric structure of the molecules could be clearly observed. The molecules protruded 2.22 ± 0.22 nm from the membrane. After removing the cAMP from solution, the MloK1 molecules underwent a drastic conformational change and the characteristic fourfold symmetric structure was lost (Figure 7 C and D). Furthermore, the molecules showed a increased height of 3.39 ± 0.85 nm.

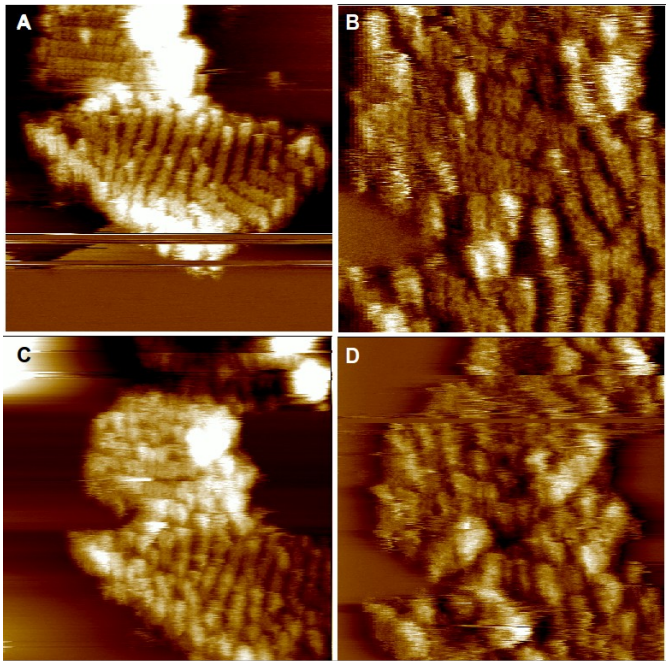


Figure 7: *MloK1 membrane in presence and absence of cAMP.* ***A)*** *AFM topograph of MloK1 in the presence of cAMP. The regular arrangement and the fourfold symmetry of the molecules is clearly observed. Scan range was 256 nm and z-scale was adjusted to 10 nm.* ***B)*** *Higher magnification of image A (128 nm scan range). Z-scale was adjusted to 5.3 nm and a gaussian flattening was applied.* ***C)*** *Same membrane patch without cAMP in the measuring solution.*

D) *Zoom-in image of 177 nm of C (Height-scale is 10 nm, gaussian flattening applied). The characteristic tetrameric structure was lost.*

Conclusion

During this COST-STSM-TD1002 mission the preparation procedure for MloK1 membranes was optimized by adjustment of protein concentrations, incubation times and the usage of EDTA. Using convential AFM imaging in contact mode single MloK1 molecules were visualized in both conformational states. While channels with bound cAMP to their CNB domain showed the characteristic tetrameric structure, the identical membrane underwent a conformational change in absence of cAMP resulting in a loss of the clear fourfold symmetry and a height increase of about 1 nm.

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