The Structure of the Prokaryotic Cyclic Nucleotide-Modulated Potassium Channel MloK1 at 16 Å Resolution

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SUMMARY

The gating ring of cyclic nucleotide-modulated channels is proposed to be either a two-fold symmetric dimer of dimers or a four-fold symmetric tetramer based on high-resolution structure data of soluble cyclic nucleotide-binding domains and functional data on intact channels. We addressed this controversy by obtaining structural data on an intact, full-length, cyclic nucleotide-modulated potassium channel, MloK1, from *Mesorhizobium loti*, which also features a putative voltage-sensor. We present here the 3D single-particle structure by transmission electron microscopy and the projection map of membrane-reconstituted 2D crystals of MloK1 in the presence of cAMP. Our data show a four-fold symmetric arrangement of the CNBDs, separated by discrete gaps. A homology model for full-length MloK1 suggests a vertical orientation for the CNBDs. The 2D crystal packing in the membrane-embedded state is compatible with the S1-S4 domains in the vertical “up” state.

INTRODUCTION

Ion channels are integral membrane proteins that control ion flow across the cell membrane. The activity of an ion channel can be modulated by secondary messenger molecules to elicit a response to variations in the cellular physiological conditions. The family of cyclic nucleotide-modulated channels belongs to this category, and it comprises the cyclic nucleotide-gated channels, known as CNG channels, and the hyperpolarization-activated and cyclic nucleotide-gated channels, known as HCN channels. CNG and HCN channels are known to be involved in several crucial physiological pathways, such as visual and olfactory sensory transduction and pacemaker activity in the heart and brain (Craven and Zagotta, 2006; Kaupp and Seifert, 2001, 2002).

The activity of CNG and HCN channels is increased by binding of ligands (cAMP and cGMP) to an intracellular cyclic nucleotide-binding domain (CNBD). Different gating mechanisms have been proposed for these channels based on the symmetry assumed by the apo and ligand-bound CNBD: (1) dimer of dimers (Kuo et al., 2003; Schumacher et al., 2001; Sun et al., 2002; Zhou et al., 2004), in which the four subunits associate and activate as independent dimers; or (2) tetramers (Jiang et al., 2002; Zagotta et al., 2003), in which the four subunits activate in either a single concerted step or in four independent steps. These models structurally correspond to a two-fold symmetric tetramer and a four-fold symmetric tetramer, respectively. In an effort to distinguish between these models, structures of the isolated CNBD have been elucidated. The isolated CNBDs of HCN2 channels form a four-fold symmetric gating ring in the presence of cAMP (Zagotta et al., 2003), while the CNBDs of MloK1 form a dimer, arguing for the existence of physiological dimers in both the absence and presence of cAMP (Clayton et al., 2004). Electrophysiological experiments on CNG and HCN channels from independent groups showed evidence for both types of association and gating: either as two dimers or as independent monomers (Liu et al., 1998; Nache et al., 2005, 2006; Ruiz and Karpen, 1997, 1999; Ulens and Siegelbaum, 2003). In order to reconcile all of these apparently contradictory data, it was proposed that the CNBDs of cyclic nucleotide-modulated channels bind ligands as two independent dimers, followed by a conformational change from two-fold to four-fold symmetry as the binding signal is relayed to the C linker for channel opening (Ulens and Siegelbaum, 2003).

As the symmetry arguments are based on biochemical and structural studies performed on isolated CNBDs of cyclic nucleotide-modulated channels, only the structure of a full-length cyclic nucleotide-modulated ion channel will reveal the true symmetry of the CNBD association in the context of the transmembrane portion of the channel. Here, we present a structural analysis of a full-length prokaryotic cyclic nucleotide-modulated K+ channel, MloK1, from the symbiotic soil bacterium *Mesorhizobium loti*. MloK1 is a homolog of eukaryotic CNG and HCN channels, it is a member of the S4 superfamily of eukaryotic
Sequence Homology of MloK1

MloK1 has a high amino acid sequence homology with eukaryotic cyclic nucleotide-modulated K+ channels, as identified by MloK1 as a good candidate for structural and functional studies on this class of ion channels (Nimigean et al., 2004). The proposed topology of MloK1 and the sequence alignment of the transmembrane domains with eukaryotic voltage-gated (Kv1.2 and Shaker) K+ channels are shown in Figure 1. The membrane portion of MloK1 has a pore region formed by helices S5 and S6, with the characteristic “GYG” signature sequence for K+ selectivity (Heginbotham et al., 1994). The other four transmembrane helices (S1–S4) are homologous to the voltage-sensor domains of voltage-gated K+ channels, and the S4 domains contain three strategically placed basic residues (Nimigean et al., 2004) (Figure 1B). The cytoplasmic portion of MloK1 consists of a conserved CNBD connected by a very short linker (~13 amino acids) to the end of the S6 helix (Nimigean et al., 2004). This linker is an abbreviated version of the extended C linker domain of eukaryotic CNG and HCN channels, which was shown to play an important role in transducing the ligand-binding signal to the channel gate (Paolelli et al., 1999).

Single-Particle Transmission Electron Microscopy

Monomeric MloK1 has a calculated molecular weight of 37 kDa. The detergent-solubilized tetrameric complex has an estimated molecular weight of ~210 kDa. Detergent-solubilized and purified MloK1 protein was imaged as a negatively stained preparation by TEM, and 14,921 single particles were manually picked with the BOXER program (Ludtke et al., 1999). Of these, 5,018 were used to generate a 3D reconstruction to a resolution of 16.3 Å (Figure 2). We imposed C4 symmetry during the single-particle reconstruction, as the 2D crystal structure indicated four-fold symmetry (see below). The reconstruction shows a channel that resembles the tetrameric Shaker K+ channel structure (Sokolova et al., 2001) composed of two parts of different sizes, separated by a ~1 nm wide region of lower density (Figure 2D). The large, square domain is hypothesized to be the detergent-surrounded, pore-containing transmembrane region with a height of 5.2 ± 0.5 nm (which approximates the thickness of the membrane) and a square side length of 8.5 ± 0.5 nm. The larger transmembrane domain resembles a four-leaf propeller with a concave region in the center. The smaller “hanging gondola”-like domain is composed of four blobs arranged around the central axis. These domains can be fit inside a square with a height of 4.0 ± 0.5 nm and a square side length of 6.0 ± 0.5 nm in the single-particle reconstruction. These domains are thought to be the CNBDs. The two domains are connected at four positions over the ~1 nm wide gap by short linkers. The shared four-fold axis passes through the model along the centers of the concave region of the large domain and the hole between the small domains. The resolution of the reconstruction was determined as 16.3 Å, based on Fourier-shell correlation (FSC) with the 0.5 criterion (Figure 2C).

Molecular Docking

Two different structures were used for the docking experiments into the calculated single-particle reconstruction. The transmembrane domain of the voltage-gated K+ channel Kv1.2 (PDB code: 2A79; [Long et al., 2005]) was used for the large domain, as Kv1.2 was proposed to be in a more “native”-like conformation than KvAP (Lee et al., 2005), due to the presence of lipids during purification and crystallization as well as the constraint of the S1 helix by the T1 domain (Lee et al., 2005). For the smaller ring-like domain, the structure of the CNBD of MloK1 was used (PDB code: 1VP6; [Clayton et al., 2004]).

The pore region of Kv1.2, consisting of helices S5 and S6, agreed well with the central region of the reconstructed MloK1 model, and it correlated well with the concave region in the center of the reconstruction model (Figure 2D). The four voltage-sensing subunits (helices S1–S4) also fit nicely into the four propeller-like regions on the MloK1 model, leaving room for a surrounding detergent belt. The loop between the pore helix and helix S5 of Kv1.2 fits the protrusions close to the four-fold center of the MloK1 reconstruction.

Docking attempts of the MloK1 CNBD dimers (PDB code: 1VP6; [Clayton et al., 2004]) into the smaller four blobs failed. Similarly, even though the four densities in our single-particle reconstruction are arranged in a square with similar outer dimensions to the ring-like structure observed by Zagotta et al. (2003), docking with a ring-like arrangement of four connected CNBDs failed (Figure 3B). However, we could dock four individual MloK1 CNBDs into the four densities of our reconstruction, resulting in a monomeric arrangement of the CNBD such that its N terminus is oriented toward the transmembrane part and the cAMP-binding pocket is facing...
vertically away from the membrane (Figure 2, and compare Figure 3A with Figure 3B). While the low resolution of our reconstruction does not allow for a safe assignment of the orientation of the CNBDs, our data document the presence of narrow gaps between the monomeric CNBDs and their four-fold symmetric arrangement.

**Electron Crystallography of 2D Membrane Crystals**

Purified MloK1 was reconstituted into phospholipid membranes and 2D crystallized in the membrane-embedded state by temperature-controlled, slow-detergent dialysis (Jap et al., 1992). 2D crystals of up to 1 μm in diameter were obtained in the presence of 200 μM cAMP. Crystals were imaged as negatively stained preparations in the TEM (Renault et al., 2006) (Figure 4A). The calculated power spectrum of recorded 2D crystal images showed clear diffraction spots up to 2 nm resolution before image unbending (Figure 4B).

Comparison of phase residuals with the ALLSPACE program (Valpuesta et al., 1994) revealed P4212 symmetry for deeply stained 2D crystals (Figures 4C and 5A), and P4 symmetry with a second, differently stained tetramer in the corners of the unit cells for unevenly stained sample preparations (Figure 5B), both at 16 Å resolution (Table 1). This indicates a head-to-tail packing of neighboring tetramers, supporting the P4212 symmetry. The unit cell parameters were a = b = 129 Å, γ = 90°. The nonsymmetrized unit cell shows a central tetrameric complex with a brighter four-fold symmetric density in the center, with
an inner diameter of 2.5 nm, and a square side length of 4.5 nm, which is surrounded by a propeller-like density of 7 nm square side length.

We interpreted the map in Figure 5A as showing both surfaces equally well stained, and the map in Figure 5B as showing one surface stained more strongly than the other surface. We therefore used the two maps to calculate a difference map, which shows the surface contours of one surface only (Figure 5C). This map revealed for each tetramer four bright, elongated densities in a propeller-like fashion. These were in good agreement with the shape and arrangement of the four densities ascribed to the CNBDs in the single-particle reconstruction (Figures 2D, 3, and 5D).

Figure 2. Single-Particle TEM Imaging of MloK1
(A) Electron micrograph of negatively stained MloK1 particles. Detergent-solubilized MloK1 is bright on a dark background. The scale bar is 50 nm.
(B) Selected class views and particle images of the MloK1 single particles. The first row shows individual particle images from three classes in columns, the second row shows the corresponding class averages, and the last row shows the p4-symmetrized 3D reconstruction density projected in the same direction. The width of the square panels is 16 nm.
(C) Fourier-shell correlation (FSC) plot of the final 3D reconstruction, indicating a resolution of 16.3 Å (arrow, 0.5 criterion [Böttcher et al., 1997]).
(D) The 3D reconstruction from 5018 single-particle images. Left, the proposed top view from the extracellular side. Center, the bottom view, showing the four CNBDs. Right, the side view. The approximate membrane plane is indicated by the dotted lines. The scale bar is 2 nm. Inside each density we show the docking of the high-resolution structures of Kv1.2 (PDB code: 2A79) and the MloK1 CNBD (PDB code: 1VP6) into the MloK1 3D reconstruction. The α helices are blue, the β strands are yellow, and the loops are green. Red balls represent K ions in the pore.
could have produced a four-fold symmetric result from two-fold symmetric unit cells that are randomly oriented in the membrane plane (either vertically or horizontally). However, two facts show that the observed four-fold symmetry is not an averaging artifact. First, the final projection map is strongly contrasted and shows excellent phase residuals up to 16 Å resolution, documenting a low variation among the contributing individual unit cell images. Second, single-particle image processing of the unit cell images of the 2D crystal, allowing each unit cell to occupy either a vertical or horizontal orientation in the image, still produced the same final four-fold symmetric projection map. This was done by transforming the 2D crystal image into a stack of single particles representing unit cells, and subjecting this to single-particle image processing. This was done without applying any symmetry and instead allowing for a 90° rotation of the individual particle images (Zeng et al., 2007). To minimize the influence of any reference, this single-particle processing was done by using a maximum likelihood algorithm, which also allowed for a higher resolution to be obtained than crosscorrelational alignment would have produced. The obtained projection map again showed four-fold symmetry (Figure S3; see the Supplemental Data available with this article online) and clearly established the four-fold symmetry of the membrane-embedded MloK1 tetramers in the presence of cAMP.

**Model Building**

Models for full-length MloK1 with the voltage-sensor paddles in the “vertical,” “tilted,” and “horizontal” states were constructed by satisfying spatial constraints by utilizing the program MODELER (Sali and Blundell, 1993). Initial model restraints were derived from the known structure of the CNBD for MloK1 (PDB code: 1VP6; Clayton et al., 2004; Zagotta et al., 2003) coupled with homology modeling by using either the predicted sequence alignment to the transmembrane domain of Kv1.2 (“vertical” state [Long et al., 2005]) or KvAP (“horizontal” state [Jiang et al., 2003] and “tilted” state [Lee et al., 2005]). Additional spatial constraints were derived from the 3D single-particle reconstruction and the 2D crystal projection map. The resulting “vertical” and “horizontal” models could both be satisfactorily docked into the 3D single-particle reconstruction; yet, the “tilted” model had two helices protruding from the 3D reconstruction (Figure S1). However, while the intermediate or “tilted” state could be oriented within the unit cell to match the electron density of the 2D projection map, such a physical arrangement would be impossible within the given unit cell parameters, as it would require the paddles from adjacent MloK1 tetramers to occupy the same space (Figure S2). Thus, only the “vertical” and the “horizontal” state models fit the electron density profile of the 2D crystal projection maps when overlaid in the unit cell configuration (Figure 6; Figure S2).
established the “vertical” (or “up”) state as the likely configuration of the voltage sensors for MloK1, as the “horizontal” configuration of KvAP might have been influenced by the absence of stabilizing lipids in the 3D crystal (Lee et al., 2005).

DISCUSSION

We have determined the 3D structure of full-length MloK1, a prokaryotic cyclic nucleotide-modulated K⁺ channel, at 16.3 Å resolution and the projection map of membrane-reconstituted MloK1 within a 2D crystal at 16 Å resolution. The structural data show a four-fold symmetric arrangement of the CNBDs in the presence of cAMP, in agreement with the symmetry observed in the HCN2 CNBD structure (Zagotta et al., 2003). In addition, the structure reveals the “native” lipid-embedded arrangement of the voltage-sensor domains, which agrees with the structure of Kv1.2 (Long et al., 2005) within the resolution of the data presented here. Although it is unclear whether the open probability of MloK1 is voltage dependent, sequence homology indicates the presence of a voltage-sensor-like domain in MloK1, which is clearly defined in the single-particle reconstruction and inferred in the projection structure. Is this a bona fide voltage sensor? MloK1 has only 3 basic residues in S4, as opposed to the 6–9 present in traditional voltage-gated channels. However, it was shown in Shaker channels that the top-most four arginines account for most of the gating charge transfer across the membrane during activation, which means that our three basic amino acids should be able to confer voltage-dependent open probability if they are strategically located and if the coupling is intact (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). Furthermore, it was recently shown that a channel from Lysteria monocytogenes with only three charges in its equivalent S4 region displays voltage-dependent open probability (Lundby et al., 2006; Santos et al., 2006), and that the MmAk channel, a potassium channel from Magnetospirillum magnetotaticum that displays high sequence homology to MloK1, is sensitive to voltage (Kuo et al., 2007).

The structure of the CNBD domain of HCN2 is also a four-fold symmetric tetramer. Although the dimensions of the HCN2 CNBD tetramer arranged horizontally (a square that is 5.9 nm wide) generally correspond to the outer diameter of the densities attributed to the MloK1 CNBD in both the single-particle reconstruction and the 2D projection structure (~6.5 nm wide, with a 2.5 nm
diameter gap), they did not satisfyingly dock in either of the two, due to the gaps between the observed CNBD densities (Figure 3B). Hence, we propose a different, vertical spatial arrangement for the CNBD gating ring of MloK1 (Figure 3A). However, due to the low resolution of the MloK1 full-length structures, the contact interface and the exact orientation of the CNBDs still need to be characterized.

One explanation for the HCN2 CNBDs having a different arrangement from MloK1 is that the latter lacks a C linker domain, and it has been shown that all contacts between individual subunits in the HCN2 CNBD tetramer are made by these C linker domains (Zagotta et al., 2003). In CNG and HCN channels, the hexahelical C linker was proposed to be the transducer domain, which senses the binding of ligands within individual subunits and then relays the signal to the gate (Gordon and Zagotta, 1995a, 1995b, 1995c; Ildefonse and Bennett, 1991; Johnson and Zagotta, 2001; Paoletti et al., 1999; Zagotta et al., 2003; Zong et al., 1998). MloK1 lacks an extended C linker region and has only a maximum of 13 amino acids in between the end of S6 and the beginning of the CNBD. Despite missing this structure, MloK1 still forms a four-fold symmetric structure and allows cyclic nucleotide-modulated ion transport through its pore, suggesting that the extended C linker is not absolutely necessary for cyclic nucleotide modulation (Nimigean et al., 2004). Due to the fact that it is a central part of gating in CNG channels, the absence of the C linker might have interesting consequences for the gating of MloK1.

Interestingly, the X-ray structures of the isolated MloK1 CNBDs suggest that they form physiological dimers with and without cAMP, in contrast with both our findings and with the four-fold symmetric tetrameric structure of the HCN2 CNBD. This suggests that the oligomeric state of the isolated MloK1 CNBD domains might have been affected by the 3D crystallization conditions (Clayton et al., 2004) as well as by the lack of the transmembrane part.
of the protein. On the other hand, the CNBDs of MloK1 as well as those of CNG and HCN channels are homologous to the bacterial transcriptional activator, CAP, and protein kinases A (PKA) and G (PKG), which are physiological homodimers (Shabb and Corbin, 1992), supporting the MloK1 dimer finding. However, the fact that the dimer interface for all of the above-mentioned proteins is different and also different from the HCN2 CNBD tetramer interface implies that the oligomerization of the CNBD-containing proteins is somewhat driven by the effector domains to which the CNBDs are attached. Only structural work on the full-length proteins can clarify the gating mechanisms and symmetry of transitions. Hence, the CNBD gating ring of MloK1 in the presence of cAMP appears to be a four-fold symmetric tetramer due partly to the strong tetramerization signal from the transmembrane domain. Our results are in contrast to the single-particle 3D reconstruction by Higgins et al. (2002), who determined the 3D structure of the heterotetrameric cGMP-gated CNG channel from bovine retina at 35 Å resolution and in the absence of ligand, and suggested that the cytoplasmic domains of that channel were arranged as a pair of dimers.

To our knowledge, our 2D membrane crystals of MloK1 show for the first time structural data for a membrane-embedded conformation of voltage-sensor domains. The difference map between an evenly and an unevenly stained 2D crystal image allowed for the visualization of the density of only one membrane-distant surface, which mainly shows the projection of the CNBDs of one side of the crystal (Figures 5A–5C). The obtained map shows four densities in a propeller-like arrangement separated by gaps, in good agreement with the CNBD densities in the single-particle 3D reconstruction (Figure 5D). The S1–S4 region of the MloK1 proteins is not individually assignable in the 2D crystal data, since the staining mostly contours the crystal surface. However, the observed maps and the dimensions of the unit cell are only compatible with the S1–S4 region being located directly above the CNBDs, as also shown in the 3D reconstruction (Figures 2D and 3).

The Kv1.2 transmembrane region could satisfyingly be docked into the transmembrane region of the MloK1 single-particle 3D reconstruction; the propeller density in the MloK1 projection map was in good agreement with the conformations of the voltage-sensor domains of Kv1.2. The single-particle reconstruction of MloK1 shows some additional density in the transmembrane region between the four (S1–S4) subunits. This density could arise from detergent molecules and copurified lipids that surround and interact with the transmembrane portion of the protein and then conglomerate in the space between the voltage sensors.

In order to analyze the conformation of the S1–S4 "paddle" domains of MloK1, we constructed homology models of MloK1 based on sequence alignment to Kv1.2, KvAP, and the MloK1 CNBD crystal structures. Models were generated such that the "paddle" orientation

### Table 1. Phase Residuals in Resolution Ranges

<table>
<thead>
<tr>
<th>Resolution Range (Å)</th>
<th>IQ = 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>All IQs</th>
<th>IQ-wght</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0–40.8</td>
<td>3.0</td>
<td>9.2</td>
<td>50.6</td>
<td>44.4</td>
<td>172.7</td>
<td>51.2</td>
<td>1.9</td>
<td>142.7</td>
<td>34.0</td>
<td>19.8</td>
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<tr>
<td>40.7–28.9</td>
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<td>10.6</td>
<td>8.6</td>
<td>22.2</td>
<td>0.0</td>
<td>158.6</td>
<td>0.0</td>
<td>74.8</td>
<td>22.4</td>
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<tr>
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<td>21.0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
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<td>31</td>
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<tr>
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<td>8.1</td>
<td>29.4</td>
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<td>25.7</td>
<td>13.7</td>
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<td>16.0</td>
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<td>4.1</td>
<td>46.9</td>
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<td>14.0</td>
<td>23.6</td>
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<td>60.3</td>
<td>44.6</td>
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<tr>
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<td>46.7</td>
<td>30.0</td>
<td>60.0</td>
<td>30.0</td>
<td></td>
</tr>
</tbody>
</table>

Overall: Phase residual = 30.630; Number of spots = 176.

Phase residuals in degrees, obtained during merging of two evenly negatively stained 2D crystal image data. Columns show resolution range in Å; IQ = 1, 2, 3, 4, 5, 6, 7, 8, intensity quotient categories of reflections (Henderson et al., 1990); All IQs, average phase residuals with equal reflection weighting; IQ-wght, average phase residual with IQ-weighting. In each resolution range, the phase residuals (upper row) and the number of reflections (lower row) are given. This table documents acceptable phase residuals up to 15.4 Å resolution. The projection map data were limited to 16.0 Å resolution.
corresponded to a “vertical” conformation, as found in the Kv1.2 structure (Long et al., 2005); a “horizontal” conformation, as found in the KvAP crystal structure (Jiang et al., 2003); or the intermediate or “tilted” conformation, as described later for KvAP (Lee et al., 2005). While all three models can fit the 3D electron density from the single-particle reconstruction at the given resolution (Figure 3; Figure S1), only the “vertical” and “horizontal” conformations could fit our 2D membrane protein crystal unit cell without causing steric hindrance (Figure 6; Figure S2). Since our 2D crystals in P4212 symmetry are in a head-to-tail packing for neighboring MloK1 tetramers, the “horizontal” conformation of the paddles would be possible, although it is generally accepted by now that this extreme, likely non-native, conformation could be the result of crystallization distortions caused by the absence of lipids (Lee et al., 2005). This identified the “vertical” configuration as the state of the paddle in our MloK1 preparation (Figures 3 and 6). Future research with cryo-electron microscopy on these crystals to achieve subnanometer resolution should elucidate the precise conformational state of MloK1 in the presence and absence of cAMP, which is currently being pursued.

In conclusion, we determined the structure of a detergent-solubilized cyclic nucleotide-modulated channel at 16.3 Å resolution by single-particle electron microscopy as well as the membrane-embedded projection map at 16 Å resolution. We found that the channel forms a four-fold symmetric tetramer in the presence of ligands, and this symmetry also extends to the CNBDs. The voltage-sensor regions are located at the periphery of the core pore-forming region and directly above the CNBDs. The
paddles of the MloK1 channel molecules were found to be in the “vertical” conformation in the 2D membrane crystals.

EXPERIMENTAL PROCEDURES

Expression and Purification

MloK1 protein was expressed and purified as previously described (Nimigean et al., 2004). In brief, the C-terminal hexahistidine-tagged MloK1-coding region from M. loti cloned into an Escherichia coli expression vector (pASK90 [Skerra, 1994]) was transformed into E. coli JM83 cells. Transformed cells were grown in Terrific Broth (TB) at 37°C, and the expression of the protein was induced for 90 min with 0.2 mM 2-mercaptoethanol (Acros Organics) when the OD600 = 1. Cells were pelleted, lysed by sonication, solubilized in 50 mM n-decyl maltopyranoside (DM, Anatrace), and purified first over a Ni-affinity column (QIAGEN) and then with gel-filtration chromatography (Superdex 200, GE). To maintain integrity and function of MloK1, 200 μM cAMP (Fluka) was present throughout the purification process. The same results were obtained when E. coli total lipids (Avanti polar lipids), solubilized in 10 mM DM, were also present during purification.

Transmission Electron Microscopy Imaging

Detergent-solubilized and purified MloK1 was diluted with buffer (100 mM KCl, 20 mM Tris-HCl, 20 mM DM, 200 μM cAMP [pH 7.6]) and adsorbed onto carbon-coated copper grids, negatively stained with 2% (w/v) uranyl formiate immediately after protein purification, blotted, distilled water washed, and air-dried. Grids were imaged in a JEOL JEM-2100F transmission electron microscope equipped with a field-emission gun (FEG), under low-dose conditions. The micrographs were recorded on a 4096 × 4096 pixel TVIPS F415 CCD camera at 200 kV acceleration voltage, 1.0–1.5 nm nominal magnification, resulting in a pixel size of 2 Å.

Single-Particle 3D Reconstruction

The SPIDER (Frank et al., 1996) and EMAN (Ludtke et al., 1999) program suites were used for processing the single-particle images. Raw images were corrected for the contrast transfer function (CTF). A total of 14,921 particle images were manually boxed with a square box size of 80 pixel width. The particle images were centered and classified by multivariate statistical analysis (MSA), and an initial model was built from randomly chosen 1,000 particle images by the common-line method to determine the particle Euler angles. This first model was resolution limited to 5 nm and was used for the generation of 40 reference projections. The reconstruction was then iteratively refined, by using only the 5,018 particles with the best correlation to the reconstruction. Particles were reference-based aligned to 190 projections, using only the 5,018 particles with the best correlation to the reconstruction and 2D projection density map was calculated. Fourier-shell correlation (FSC) of successive iterations showed no further resolution improvement after six iterations (data not shown). The final reconstruction showed a resolution of 16.3 Å, as determined by FSC (using the 0.5 criterion) between reconstructions made from even and odd particles that had been aligned onto the same reference projection set (Figure 2).

Model Fitting

The atomic coordinates of Kv1.2 (PDB code: 2A79) and CNBD with ligand of MloK1 (PDB code: 1VP6) were used for docking into the MloK1 3D map. The UCSF CHIMERA graphical package (Pettersen et al., 2004) was used for the manual docking. The docking criterion was supported in the fit models in map function built into the package. The MloK1 CNBDs were tentatively tetramerized according to the atomic coordinates of the HCN2 CNBDs (PDB code: 1QSO, residues 501–633); the rmsd of the tetramerized MloK1 CNBDs and HCN2 CNBDs was 1.60 Å. However, the resulting tetrameric structure did not fit the single-particle 3D reconstruction.

2D Crystallization and Electron Crystallography Imaging

Detergent-solubilized and purified MloK1 was mixed with E. coli total lipid extract at a lipid-to-protein ratio of 0.5 and dialyzed against detergent-free buffer (20 mM KCl, 1 mM BaCl2, 1 mM EDTA, 20 mM Tris [pH 7.6]) and in the presence of 200 μM cAMP. Following recommendations from Tom Walz (Harvard Medical School), a temperature profile oscillating five times between 37°C and 25°C was used during a 5 day dialysis procedure. Grids of 2D crystals were prepared and imaged as described above. Recorded images were processed with the 2dx software package (Gipson et al., 2007), which is based on the MRG software (Crowther et al., 1996).

Homology Modeling

Full-length models of MloK1 were constructed with the program MODELER 9v1 (Sali and Blundell, 1993). Sequence alignment of MloK1 against Kv1.2, KvAP, and the known structure of MloK1’s CNBD provided the initial restraints. The atomic coordinates of the CNBD of MloK1 (PDB code: 1VP6) combined with those of Kv1.2 (PDB code: 2A79) for the transmembrane domain were used as the template for the “vertical” state model, while both the “tilted” and “horizontal” state models used the atomic coordinates of KvAP (PDB codes: 2A0L and 1ORQ, respectively) for the transmembrane domain sequence homology. Additional information from the above-mentioned 3D single-particle reconstruction and the 2D projection map, including unit cell parameters, electron density distribution, and symmetry, were incorporated as further spatial constraints to optimize the output models. The UCSF CHIMERA graphical package (Pettersen et al., 2004) was used for visualization, model fitting (as described above), and unit cell evaluation.

Supplemental Data

Supplemental Data include (1) homology models of MloK1 with the voltage sensors in either the “tilted” or “horizontal” configuration docked into the single-particle reconstruction and 2D crystal packing, and (2) the description of the single-particle image processing with a maximum likelihood algorithm and its application for the MloK1 2D crystal and are available at http://www.structure.org/cgi/content/full/15/9/1053/DC1/.

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**Accession Numbers**

The single-particle 3D reconstruction volume of full-length MloK1 has been deposited in the Electron Microscopy Data Bank (EMDB, http://www.ebi.ac.uk/msd) under accession number 5548.