

K⁺ Channels: Gating Mechanisms and Lipid Interactions



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Ralf

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Abstract

Computational methods, including homology modelling, *in-silico* dockings, and molecular dynamics simulations have been used to study the functional dynamics and interactions of K⁺ channels. Molecular models were built of the inwardly rectifying K⁺ channel Kir2.2, the bacterial homolog K⁺ channel KirBac3.1, and the twin pore (K2P) K⁺ channels TREK-1 and TRESK. To investigate the electrostatic energy profile of K⁺ permeating through these homology models, continuum electrostatic calculations were performed. The primary mechanism of KirBac3.1 gating is believed to involve an opening at the helix bundle crossing (HBC). However, simulations of Kir channels have not yet revealed opening at the HBC. Here, in simulations of the new KirBac3.1-S129R X-ray crystal structure, in which the HBC was trapped open by the S129R mutation in the inner pore-lining helix (TM2), the HBC was found to exhibit considerable mobility. In a simulation of the new KirBac3.1-S129R-S205L double mutant structure, if the S129R and the S205L mutations were converted back to the wild-type serine, the HBC would close faster than in the simulations of the KirBac3.1-S129R single mutant structure. The double mutant structure KirBac3.1-S129R-S205L therefore likely represents a higher-energy state than the single mutant KirBac3.1-S129R structure, and these simulations indicate a staged pathway of gating in KirBac channels. Molecular modelling and MD simulations of the Kir2.2 channel structure demonstrated that the HBC would tend to open if the C-linker between the transmembrane and cytoplasmic domain was modelled helical. The electrostatic energy barrier for K⁺ permeation at the helix bundle crossing was found to be sensitive to subtle structural changes in the C-linker. Charge neutralization or charge reversal of the PIP₂-binding residue R186 on the C-linker decreased the electrostatic barrier for K⁺ permeation through the HBC, suggesting an electrostatic contribution to the PIP₂-dependent gating mechanism. Multi-scale simulations determined the PIP₂ binding site in Kir2.2, in good agreement with crystallographic predictions. A TREK-1 homology model was built, based on the TRAAK structure. Two PIP₂ binding sites were found in this TREK-1 model, at the C-terminal end, in line with existing functional data, and between transmembrane helices TM2 and TM3. The TM2-TM3 site is in reasonably good agreement with electron density attributed to an acyl tail in the recently deposited TREK-2 structure.

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Publications

A number of publications resulted from the research work on K^+ ion channels reported in this thesis. Chapter 3 to 5 are largely based on published articles, as detailed below.

Vassiliy N. Bavro [†], Rita de Zorzi [†], Matthias R. Schmidt, João R.C. Muniz, Lejla Zubcevic, Mark S.P. Sansom, Catherine Vénien-Bryan, Stephen J. Tucker, 2012. Structure of a KirBac Potassium Channel with an Open Bundle-Crossing Indicates a Mechanism of Channel Gating. *Nature Structure and Molecular Biology* **19** (2) 158–163.

An *in-silico* mutation in the helix bundle of the new, apparent open, S129R crystal structure of the KirBac3.1 K^+ channel results indeed in an open channel, demonstrating that rotation of the cytoplasmic domain leads to opening at the helix bundle crossing (Chapter 3). Featured in *PSI Nature* "Research Advances section" (doi: 10.1038/sbkb.2011.66).

Matthias R. Schmidt, Phillip J. Stansfeld, Stephen J. Tucker, Mark S.P. Sansom. Simulation-Based Prediction of Phosphatidylinositol 4,5-Bisphosphate Binding to an Ion Channel, 2012. *Biochemistry* **52** (2) 279–281.

This article validates the multi-scale method for the prediction of PIP₂ binding to an ion channel (Chapter 4). This article was chosen by the editor to be highlighted on the journal's web site.

Markus Rapedius[†], Matthias R. Schmidt[†], Chetan Sharma, Phillip J. Stansfeld, Markus Rapedius, Mark S.P. Sansom, Thomas Baukrowitz, Stephen J. Tucker, 2012. State-Independent Intracellular Access of Quaternary Ammonium Blockers to the Pore of TREK-1. *Channels* **6** (6) 473–478.

The selectivity filter of TREK-1 has been implicated in gating, but recent crystal structures have suggested an alternative access pathway for channel blockers, suggesting that the helix bundle crossing (HBC) may form an alternative gate. This article rules out the possibility that TREK-1 is gated at the HBC (Chapter 5).

Lejla Zubcevic, Vassiliy N. Bavro, João R.C. Muniz, Matthias R. Schmidt, Shizhen Wang, Rita de Zorzi, Catherine Vénien-Bryan, Mark S.P. Sansom, Colin G. Nichols and Stephen J. Tucker. Control of KirBac3.1 potassium channel gating at the interface between cytoplasmic domains. *Journal of Biological Chemistry* **289** (1) 143–151.

This article demonstrates the effect of a cytoplasmic mutation on activation gating in KirBac3.1 (Chapter 3).

[†] denotes joint first authorship.

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Chapter 1

Background

1.1 Membrane proteins

Membrane proteins play key roles in many aspects of cellular function. Encoded by 20–25 per cent of all human genes [1], more than 50 per cent of membrane proteins form targets of molecular therapy [2]. Ion channels are pore-forming proteins and account for an important sub-class of membrane proteins [3]. Their primary role is to control the distribution of potassium (K^+), chloride (Cl^-), sodium (Na^+) and calcium (Ca^{2+}) ions on both sides of the cell membrane, which is otherwise impermeable for the passage of charged, hydrophilic molecules. The existence of four hundred putative ion channel genes, according to the human genome project, and over two hundred human ion channel proteins according to the International Union of Basic and Clinical Pharmacology (IUPHAR), demonstrates the diversity and complexity of the ion channel protein family [4]. In addition to regulating the electrical signal transduction in excitable cells [5], ion channels also control such diverse physi-

ological processes as the cytoplasmic cell volume, intra- and extracellular pH, ion transport across epithelial cell membranes, and chemical Ca^{2+} signalling [6]. Consequently, more than sixty channelopathies [7], human diseases causally linked to inheritable mutations in ion channel genes, have drawn considerable resources to the study of ion channel function, structure and pathophysiology [8].

1.2 K^+ ion channels

Diseases linked to dysfunction of K^+ channels are diabetes, Bartter's syndrome, Long-QT syndrome, episodic ataxia, epilepsy, pain perception, depression and migraine, to name a few [9, 10]. These diseases affect ion permeation across cell membranes and thereby essential electrical properties of nerve and muscle cells. Normally, entry of Na^+ ions depolarises the cell membrane, and the outflow of K^+ ions through K^+ -selective ion channels out of the cell subsequently repolarises the cell membrane, restoring the cell's excitability. This mechanism establishes the action potential and triggers signal transmission in neurons or contraction of muscle cells such as ventricular myocytes. In the long QT syndrome disease state, the duration of the time period between depolarisation and repolarisation is prolonged, due to a dysfunction of K^+ channels. Consequently, the inhibited outflow of K^+ ions during repolarisation of ventricular myocytes may lead to potentially fatal cardiac arrhythmias such as the Torsade de pointes arrhythmia.

This thesis is concerned with K^+ ion channels, particularly with inwardly rectifying (Kir) K^+ channels encoded by *KCNJ* genes [11], and with K^+ chan-

nels characterized by two pore domains (K2P), encoded by *KCNK* genes [12, 13]. With 92 human K^+ genes [14], K^+ channels belong to the most abundant and diverse class of ion channels to date [15, 16]. K^+ channels, following the IUPHAR nomenclature (<http://www.iuphar-db.org>), belong to the 140 member strong superfamily of voltage-gated ion channels [17, 18], which exists next to the 70 member strong ligand-gated ion channel superfamily (recently reviewed by Lemoine *et al.* [19]). Other anion channels are described in several online curated databases of the entire ion channel family (see, for example, the Guide to Pharmacology database, <http://www.guidetopharmacology.org>).

1.2.1 Biophysical properties of K^+ channels

Different ion concentrations in the intra- and extracellular space establish a transmembrane potential, such that at negative transmembrane voltages, the net charge of all ions is more negative inside than outside the cell membrane (see Hille *et al.* [5]). At the resting state of the cell (approximately -70 mV), the net ion flow through the cell membrane is zero. Electrical excitation of the cell changes intra- and extracellular ion concentrations such that the transmembrane voltage turns positive, and the cell depolarizes, triggering an action potential. The Nernst equation describes the transmembrane potential at which the net flow of ions through a set of ion channels would be zero, if there was only one species of permant ions and one corresponding population of ion channels. This potential is called the reversal potential, the equilibrium potential, or the Nernst potential of the ion:

$$V_K = \frac{RT}{ZF} \ln \frac{[K^+]_o}{[K^+]_i} \quad (1.1)$$

where V_K is the equilibrium potential (V), R is the universal gas constant ($\text{J K}^{-1} \text{mol}^{-1}$), T is the absolute temperature (K), Z is the valence of the permeant ion, F is the Faraday constant (As mol^{-1}), $[K^+]_o$ is the extracellular concentration of the permeant ion (M) and $[K^+]_i$ is the intracellular concentration of the permeant ion (M). Here, the nomenclature is chosen such that the permeant ion represents K^+ . The Nernst equation is useful to interpret electrophysiological current-voltage (I-V) measurements. For example, a change in the reversal potential indicates a change in the effective ion concentration, and thus a change in channel selectivity.

For multiple (monovalent) ion species, such as K^+ , Na^+ and Cl^- , the Goldman-Hodgkin-Katz (GHK) equation, a generalization of the Nernst equation, describes the resting membrane potential at which the total ion current through the membrane vanishes. A derivation can be found in Zaydman et al. [20], based on Fick's law of diffusion and on Ohm's law of electricity [21, 22]. The resting membrane potential is the sum of the equilibrium potentials of each ion species weighted by the relative permeability of the membrane to those ions:

$$V_{rest} = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \quad (1.2)$$

Here, V_{rest} is the resting (steady-state) membrane potential (V), R is the universal gas constant ($\text{J K}^{-1} \text{mol}^{-1}$), T is the absolute temperature (K), Z is the valence of the permeant ion, F is the Faraday constant (As mol^{-1}), $[K^+]_o$ is the extracellular K^+ concentration (M), $[K^+]_i$ is the intracellular K^+ concentration (M) and P_K is the relative permeability of the ion channel to K^+ . The permeabil-

ity and the intra- and extracellular concentrations for Cl^- and Na^+ are named correspondingly.

The GHK equation assumes that the ion channel is non voltage-dependent, as well as highly selective and diffusion-limited. In other words, the ion channel can occupy only two states, either open or closed, the conductivity in the open state is constant, and the ion channel probability of being in an open state is independent from the transmembrane potential. The result is a linear I-V relationship, an ion channel property named GHK constant field rectification [23]. For a homogenous subpopulation of ion channels following the GHK rectification, the macroscopic current amplitude is proportional to the number of ion channels, their conductivity, open probability and to the difference between the membrane potential and the equilibrium potential of the permeant ion:

$$I(V_m) = N\gamma P_o(V_m - V_K) \quad (1.3)$$

The magnitude of $I(V_m)$ determines the macroscopic current I (A) at the transmembrane voltage V_m (V). N is the number of (K^+) ion channels, γ the conductance (S) of a single open (K^+) channel, P_o the probability of the ion channel of being open and V_K is the equilibrium potential (V) for the ion (K^+).

These are the defining biophysical fundamentals of ion channels. The next section will introduce some important examples of K^+ channels that illustrate the structural and functional diversity of the K^+ ion channel family.

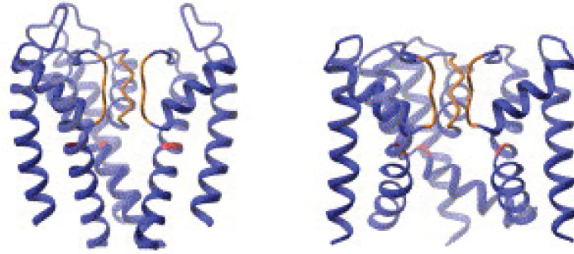


Figure 1.1: Closed and opened conformations of the pore. The selectivity filter sequence is highlighted orange and the glycine gating hinge red. Three subunits of the closed pore conformation of KcsA (left) and the opened pore conformation of MthK (right) are shown with the selectivity filter and gating hinge coloured. Figure and caption reprinted from [24], © (2003), with permission from Elsevier.

1.2.2 K^+ channel family and structures

The cloning of the first *Shaker* K^+ channel [25] from *Drosophila melanogaster* has been a milestone in the study of the electrophysiological properties of K^+ channels. K^+ channels are historically classified by the type of intra- or extracellular signals that change their open probability. These signals could be ligands, such as Ca^{2+} , pH, ATP, PIP_2 , changes in the transmembrane potential, or entire proteins (G-proteins are released from the intracellular membrane interface of the G-protein coupled receptors, following stimulation by neurotransmitters or hormones, and are known to activate certain K^+ channels).

However, it took more than a decade until the first K^+ channel structure was determined, from the voltage-dependent KcsA channel from *Streptomyces lividans* [26]. The calcium-dependent K^+ channel from the bacterium *Methanobacterium thermoautotrophicum* was crystallised later in 2002 [27, 28] (Figure 1.1). Today, several structures of eukaryotic voltage-gated K^+ and bacterial Na^+ channels [29], and of bacterial Ca^{2+} (MthK)- or cyclic-nucleotide (MlotiK)-gated

K^+ channels have been determined. The bacterial structures provide models, which have been used to study the mechanism of K^+ channel gating, in the tacit assumption that this would be applicable to eukaryotic ion channels. A comprehensive list of K^+ channel structures can be found in the database of membrane proteins of known structures by S. White's laboratory at the University of California, Irvine (<http://blanco.biomol.uci.edu/mpstruc/>).

For this study of the gating mechanism and of the lipid interactions of K^+ channels, the most important structures are the eukaryotic Kir and K2P channel structures (Figure 1.2), which reveal intra- or extra-cellular domains (Figure 1.3). The intracellular domain of Kir channels binds molecules or proteins that gate the channel open or closed (G-proteins, Na^+ , ATP, PIP_2). For K2P channels, the role of the extracellular cap domain is still obscure. The linear I-V relationship predicted by the GHK equation is compatible with K2P channels, but inwardly-rectifying (Kir) K^+ channels, which are more conductive for K^+ owing into than out of the cell, deviate from this relationship at positive transmembrane potentials [30]. K2P channels are sometimes known as background channels, or leak K^+ channels.

Despite this structural and functional diversity, all K^+ channels possess a highly conserved pore-forming selectivity filter structure. Permeant ions need to shed their hydration shell in order to pass the selectivity filter, and the associated energy cost of dehydration is compensated for by the interaction of the permeant ion with the carbonyl atoms of the filter. Only for K^+ ions, is this balance between dehydration energy and carbonyl interaction favorable sufficient to allow conduction [31, 32]. Therefore, all K^+ channels are highly selective for K^+ at nearly diffusion-limited conduction rates ($> 10^6 \text{ sec}^{-1}$) and are able to

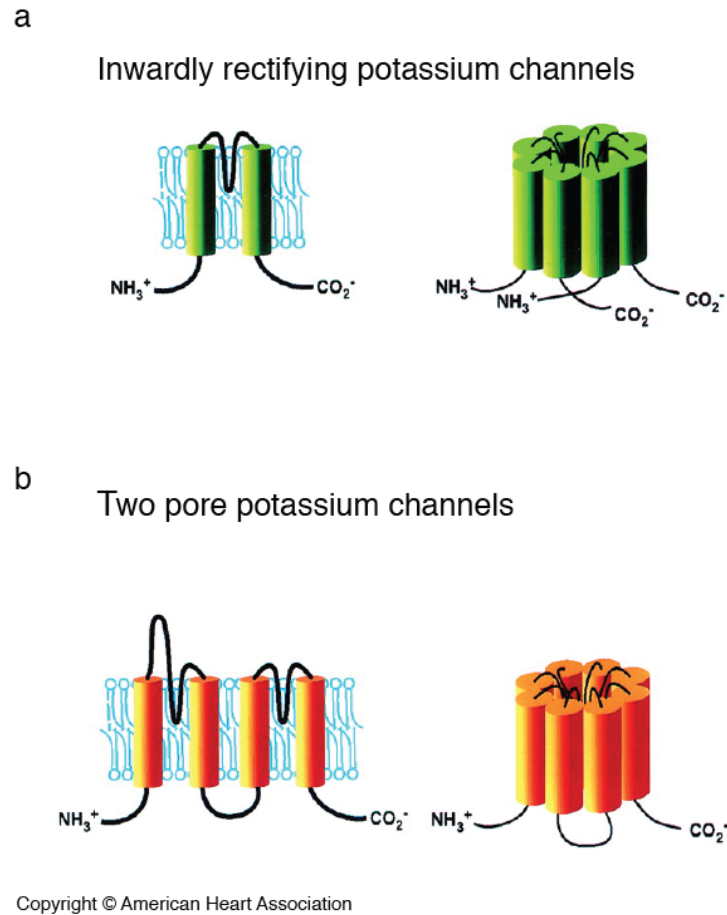


Figure 1.2: Molecular topology of inwardly-rectifying (Kir) and two pore domain (K2P) K^+ channels. The lipid bilayer is indicated. (a) Kir channels are tetramers with one pore forming domain per subunit and two transmembrane helices. (b) The arrangement of K2P channels is fundamentally different from Kir channels. Four transmembrane helices and two pore domains assemble as dimer. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: Circulation Research [33], (© 2001).

select K^+ ions (atomic radius of $1.33 \text{ \AA}$) over Na^+ ions (atomic radius of $0.95 \text{ \AA}$) by factor of more than a thousand [24].

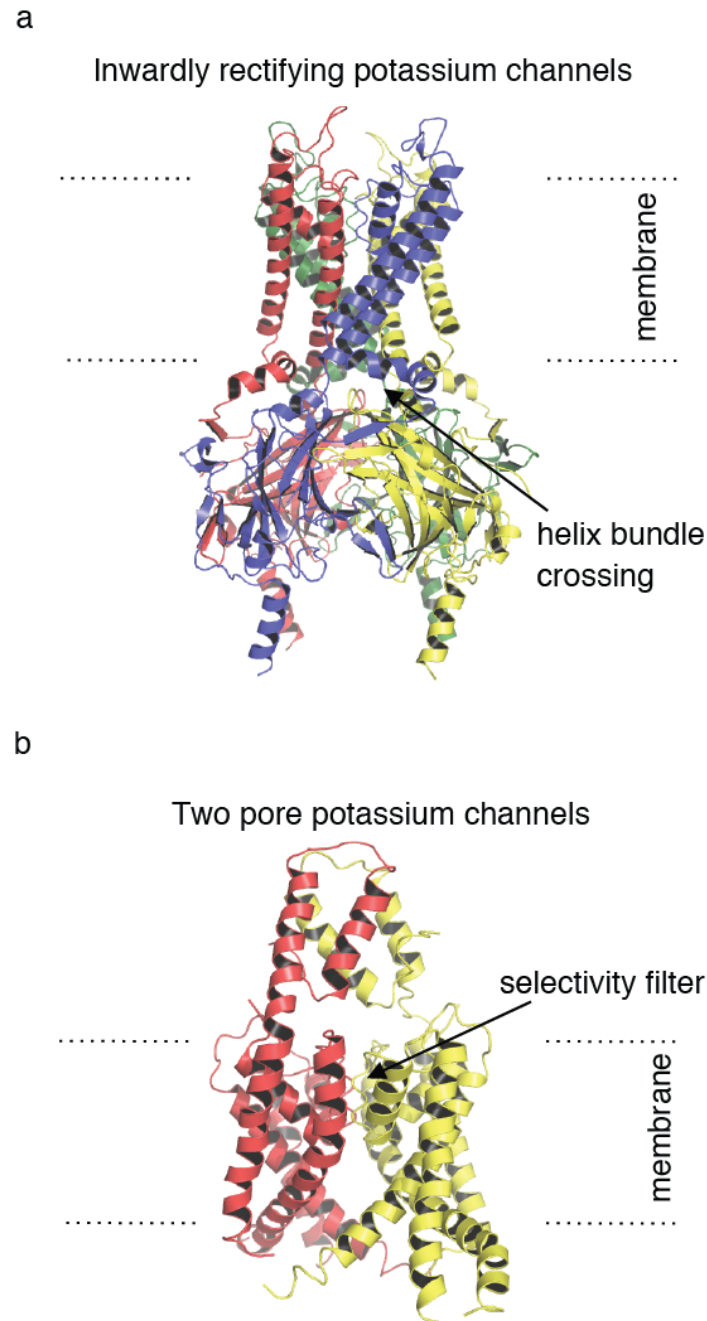


Figure 1.3: Structure of inwardly-rectifying (Kir) and two pore domain (K2P) K^+ channels. The lipid bilayer is indicated. (a) The primary gate of Kir channels is believed to be at the helix bundle crossing. (b) It is suggested that K2P channels gate near the selectivity filter [34]. Coloured by subunit (red, green, blue, yellow). All protein structures in this thesis were drawn with PyMOL [35], VMD [36] or Chimera[37].

1.2.3 K^+ channel gating

Gating denotes the process by which ion channels transition between open and closed states. The mechanism of ion channel gating is often coupled to conformational changes [38]. The first structural view on gating of K^+ channels was obtained by comparing the structure of the prokaryotic K^+ channel KcsA from *S. lividans* [26, 39, 32] with the open crystal structure of the prokaryotic K^+ channel MthK from *M. thermoautotrophicum* [27, 28] (Figure 1.1). This has led to the current hypotheses of K^+ channel gating [40]. Kir channels are believed to primarily gate by opening and closing a constriction at the helix bundle crossing (Figure 1.3a), while K2P channels are predicted to gate by changing the conductivity of the selectivity filter [41] (Figure 1.3b).

The conductance of K^+ channels when open is primarily determined by the properties of the ion permeation pathway and the channel pore, while the probability of the ion channel of being open is subject to regulation by intra- and extracellular signals, such as pH, pressure, temperature, membrane stretch, and phosphorylation [20]. Of course, this is only an approximation. Multiple conductance states are possible, and gating and permeation are not always independent [42, 43]. However, this model of regulation will be used to explain the gating mechanisms that are studied and discussed in the following chapters.

1.2.4 Inwardly rectifying K^+ (Kir) channels

Kir channels allow K^+ to move more easily into than out of the cell [30, 44] and serve diverse physiological functions. Inheritable mutations in Kir channel genes lead to deafness [45], angina [46], congenital diabetes [47, 48], and

epilepsy [49]. The Kir channel family (Figure 1.4a) comprises seven subfamilies, whose function and regulation can be classified in four groups: the Kir2 channels, which are constitutively active; the G-protein dependent Kir3 channels, which are activated by receptor stimulation; ATP sensitive Kir6 channels, which couple the metabolic state of the cell to hormone secretion; and other Kir channels (Kir1, Kir4, Kir5, Kir7) which are primarily involved in K^+ transport across non-excitabile endothelial, epithelial or glial cells. This section provides some examples of the physiological functions and a summary of the structural features of those Kir channels referred to in this thesis.

Kir channel physiology

Kir channels are expressed in a wide variety of excitable (neurons, muscle cells, cardiac myocytes) and non-excitabile cells (epi- and endothelial cells, glial cells, blood cells), and their physiological function is therefore complex. Kir2 channels play an important role in maintaining the resting membrane potential in the heart and are strongly expressed in the His bundle, Purkinje fibres, and ventricular myocytes. Kir2.1 knockout mice were found to have a slower heart rate [11]. Andersen's syndrome is an inheritable disorder characterised by cardiac arrhythmia, dysmorphic bone features, and periodic paralysis [11]. The disease is linked to loss-of-function mutations in the Kir2.1 channel, where some mutations are known to inhibit Kir2.1-PIP₂ interactions [50], while others inhibit trafficking of Kir2.1 to the membrane [11]. Mutations in Kir2.1 responsible for Andersen's syndrome suppressed the inward-rectifying I_{K1} current in rat myocytes and lead to distorted cardiac action potentials and ventricular arrhythmias [51]. Activation of Kir3 channels slows the heart rate upon nervus vagus

and G protein coupled receptor (GPCR) stimulation [52]. For a comprehensive overview, see the reviews by Nichols *et al.* or Hibino *et al.* [53, 11].

Kir channel structure

Towards the outset of this thesis (2010), knowledge of full-length Kir channel structures was limited to X-ray crystal structures of bacterial Kir (KirBac) channels [59, 60] and to structures obtained by 2D electron crystallography [61, 62, 63]. Despite differences in the residues that interact with highly charged anionic lipids, KirBac and Kir channels share the same overall architecture. Kir channels are characterized by a long continuous pore, which comprises a vestibule in the centre of a large cytoplasmic domain, and a cavity in the transmembrane domain that leads to a highly conserved K^+ selectivity filter (Figure 1.4b). However, all these structures were crystallized in the closed state. Here, on the basis of an *in-silico* mutation in the X-ray structure of mutant (closed) KirBac3.1 from *M. magnetotacticum*, and with molecular dynamics simulations of this structure, it will be shown that KirBac3.1 gates by opening a constriction at the helix bundle crossing (Chapter 3). This supports the more conventional model of gating and contradicts an alternative hypothesis by which changes in the cytoplasmic domain of KirBac3.1 are directly coupled to the conductivity of the selectivity filter [64].

A year later, in 2011, Hansen *et al.* determined the first lipid-bound Kir2.2 structure from *Gallus gallus* [58] (a structure without bound lipids was solved in 2009 [65]). These Kir2.2 structures suggested a gating model for Kir channels by which binding of a highly negatively charged lipid leads to conformational changes at the helix bundle crossing. In Chapter 4, it will be shown that the

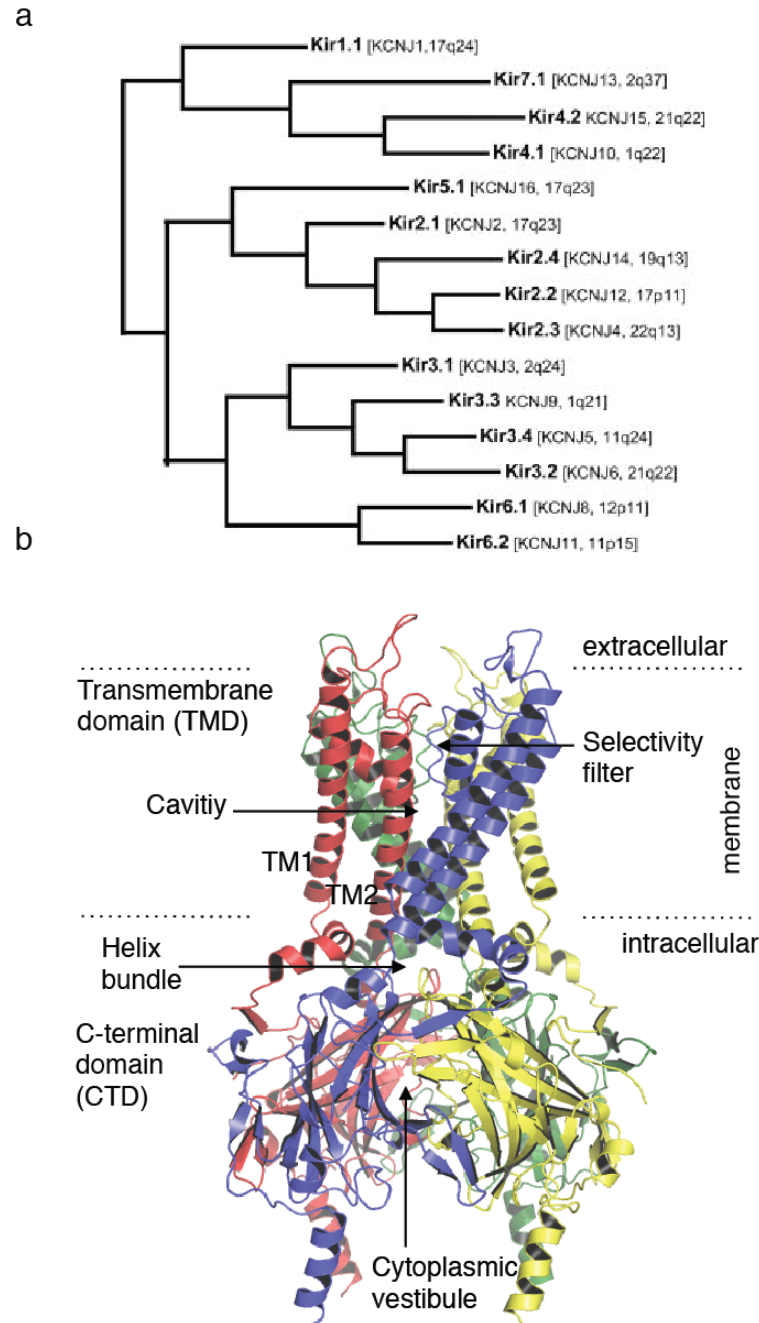


Figure 1.4: Phylogenetic tree and structure of human Kir channels. **(a)** Phylogenetic tree for 15 known members of the human Kir family. Kir2.6, which “shares 99 per cent identity with Kir2.2” [54], is not shown. Recently identified channels ccKir2.5 (71 per cent identity with mammalian Kir2.2) and zKir6.3 (66 per cent identity with mammalian Kir6.2) were only found in crucian carp and zebrafish, respectively [55, 56]. International Union of Pharmacology and HUGO Gene Nomenclature Committee names of the genes are shown together with their chromosomal location. Adapted with permission from The American Society for Pharmacology and Experimental Therapeutics: Pharmacological Reviews [57]. **(b)** X-ray crystal structure of Kir2.2 [Protein Data Base (PDB) id: 3SPI] [58], coloured by subunit.

electrostatic energy profile of the Kir2.2 channel is highly sensitive to structural changes in this lipid binding site.

1.2.5 Two pore domain K⁺ (K2P) channels

The two pore domain weakly inward-rectifying (TWIK)-related K⁺ channel (TREK channel) and the TWIK-related spinal cord K⁺ channel (TRESK channel) [66, 67] are the most active background potassium channels in the dorsal root ganglion neurons [68, 69], where they are responsible for the stabilization of the negative resting membrane potential and thereby for the regulation of cellular electrical excitability [12, 70]. They form part of the 15 member twin pore channel (K2P) family, which contributes to the background leak current of the action potential, but whose members are regulated by distinct mechanisms [71] (Figure 1.5a). TREK-1 and TRESK were shown to be involved in a number of neurological disorders [72, 73]. Deletion of TREK-1 produced a depression-resistant phenotype with a reduced sensitivity to volatile anesthetics [72, 74]. TREK-1 knockout mice, with the *KCNK2* gene not expressed, are insensitive to halothane and chloroform [75]. TREK-1 is also implicated in neuroprotection [74, 76]. Moreover, expression levels of TREK-1 have been correlated with proliferation of cancerous prostate cells, and with cancer progression in mice [77].

The TREK-1 channel is a member of the mechano-sensitive K2P subfamily comprising TREK-1, TREK-2 [78] and TRAAK (TWIK-related arachidonic acid activated K⁺ channel [79]. TREK-1 shares 63 per cent and 45 per cent sequence identity with TREK-2 and TRAAK, respectively, but unlike TRAAK, is also expressed outside of the nervous system [80]. TREK-1 expression patterns

and single-channel characteristics depend on the isoform; if the N-terminus is truncated in an alternative initiation of translation, TREK-1 displays increased permeability to sodium [81], however this effect has not been confirmed yet.

In the case of TRESK, a mutation in gene *KCNK18* that truncates the channel prematurely has been found to cause migraine with aura [82], a common and often debilitating medical condition with a lifetime prevalence of 3 per cent in men and 10 per cent in women [83, 84], leading to severe, long-lasting headaches, preceded by sensory warning symptoms or visual disturbances. This finding provided direct evidence that migraine is linked to cellular excitability and suggested that selective activators of TRESK channels may form potential targets for the symptomatic and prophylactic treatment of migraine with aura [82, 85].

Structure

The structure of K2P channels is unique amongst the K^+ channel family. While Kir channels form a channel pore by assembling as tetramers of four usually identical subunits [65, 88], the K2P channel pore is formed by two subunits, which dimerise into a pseudotetrameric pore [89]. No crystal structures of K2P channels were available when the work on this thesis was started, but the dimerisation of K2P subunits suggested an asymmetry in the pore-lining helices (Figure 1.5b). To date, three K2P channel crystal structures have been determined, displaying an asymmetric cavity below the selectivity filter [90, 86, 91, 92]. However, despite these structural advances, regulatory sensors of pH and basic lipid-interacting amino acids in TREK-1 are part of the yet unresolved cytoplasmic domain [93, 94, 95]

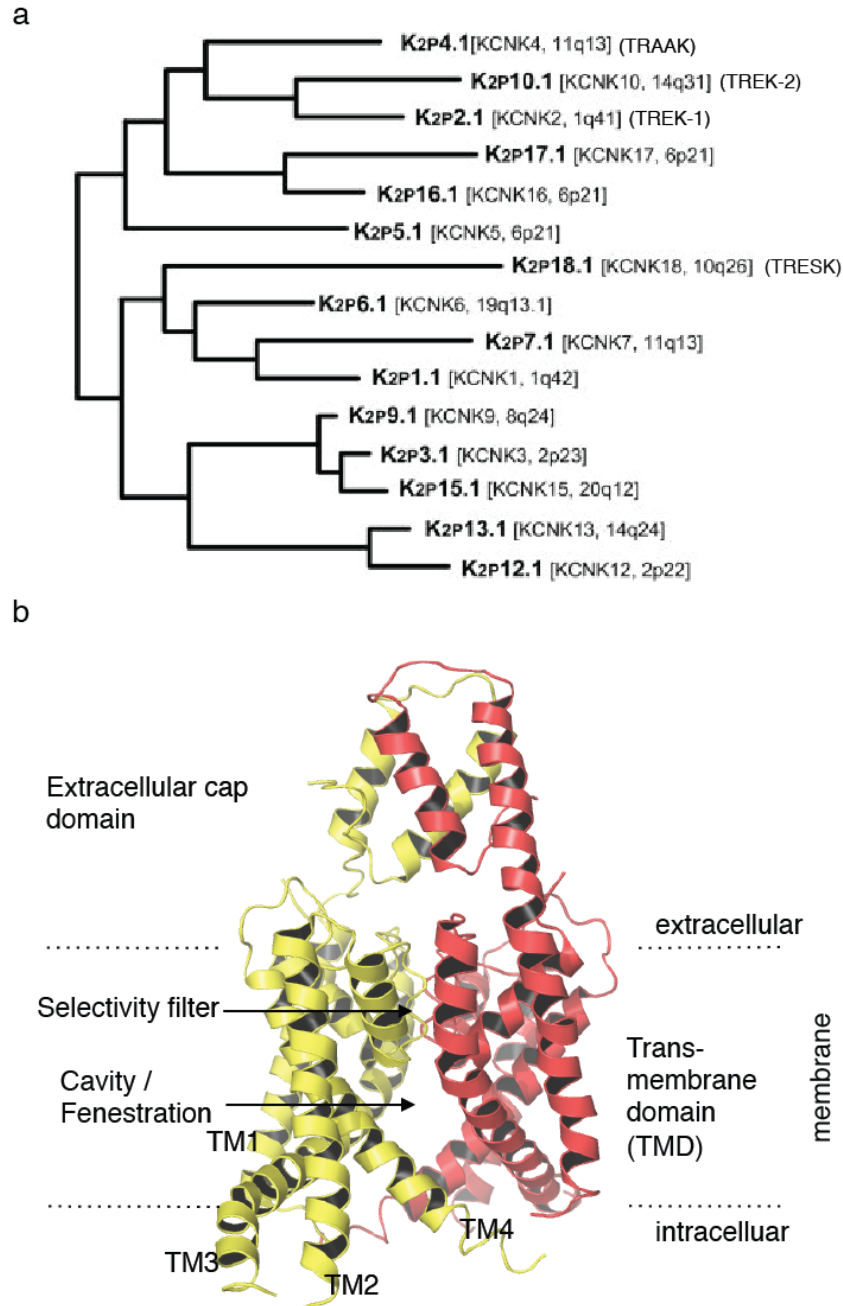


Figure 1.5: (a) Phylogenetic tree of 15 known human K2P channels and (b) structure of K2P4.1 (TRAAK), PDB id: 3UM7 [86], coloured by subunit. (a) Adapted with permission from The American Society for Pharmacology and Experimental Therapeutics: Pharmacological Reviews: [87]

Regulation

TREK-1 is regulated through stretch, fatty acids, intra- and extracellular pH, heat and through stimulation of Gq protein-coupled receptors (GqPCR) [96, 95] (Figure 1.6). The TREK-1 C-terminal domain comprises key residues which are sensitive to phosphorylation (S300 and S333) and pH (E306). A Kinase anchoring protein 150, an intracellular scaffolding protein that binds between V298 and R311 on the C-terminal domain, reduces the sensitivity to GqPCR-mediated inhibition [97, 98, 99].

TRESK is activated by the binding of the Ca^{2+} dependent phosphatase calcineurin to a nuclear factor of activated T-cells (NFAT)-like binding motif, following stimulation of GqPCRs, such as the M_3 muscarinic acetylcholine receptor, and the rise of intracellular Ca^{2+} concentration [100]. The subsequent dephosphorylation of serine residues S252, S262, S264, and S267 in the large intracellular regulatory domain of TRESK between the transmembrane (TM) helices TM2 and TM3 [101, 102] is thought to stabilise the open state of the channel, by exerting forces on TM2 and TM3. Indeed, the S276A and S252A mutations render TRESK constitutively open [103]. That and the role that TRESK is suggested to play in calcineurin inhibitor-induced pain syndrome [104], along with the activation of TRESK by volatile anesthetics such as halothane [105], suggests an involvement of TRESK in pain pathways [106, 107].

Pharmacology

It is important to understand that K₂P channels are elements of complex signalling networks (Figure 1.6) [108]. Drugs that interfere with signalling path-

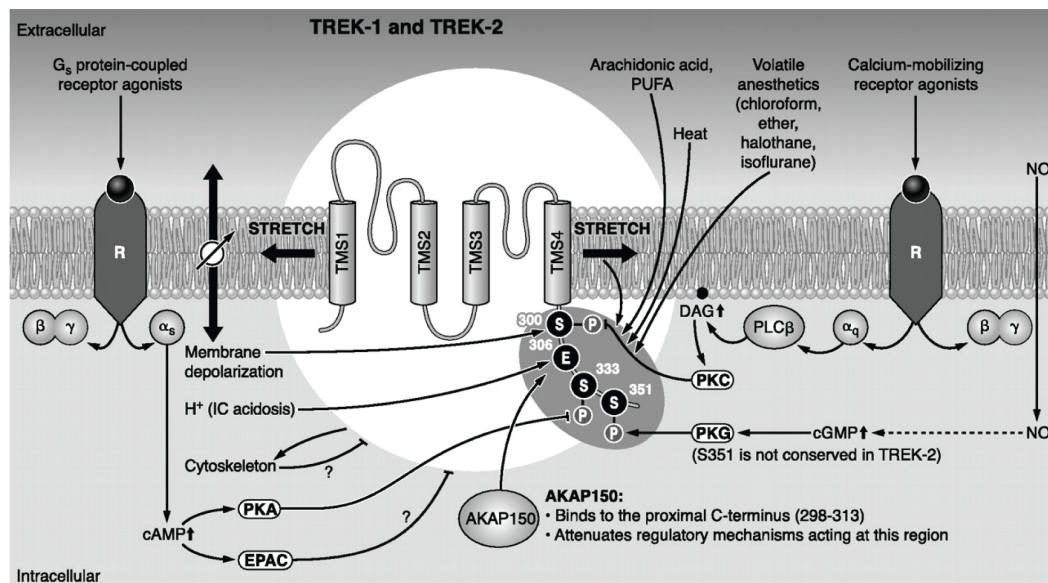


Figure 1.6: Regulation of TREK channels. Polymodal control of TREK-1 (and TREK-2). TREK-1 is activated by physicochemical changes, such as stretch or convex deformation of the plasma membrane, depolarization, heat, and intracellular acidosis. Polyunsaturated fatty acids (PUFA) including arachidonic acid (AA) and a wide variety of volatile anesthetics also open TREK channels. Activation of the G_s/cAMP/PKA and the G_q/PLC/DAG/PKC signalling pathways inhibit TREK channels by phosphorylating defined serine residues on the C-terminal tail. Arrows indicate stimulation; lines with T ending represent inhibition. Adapted with permission from The American Physiological Society: Physiological Reviews [12] (© 2010).

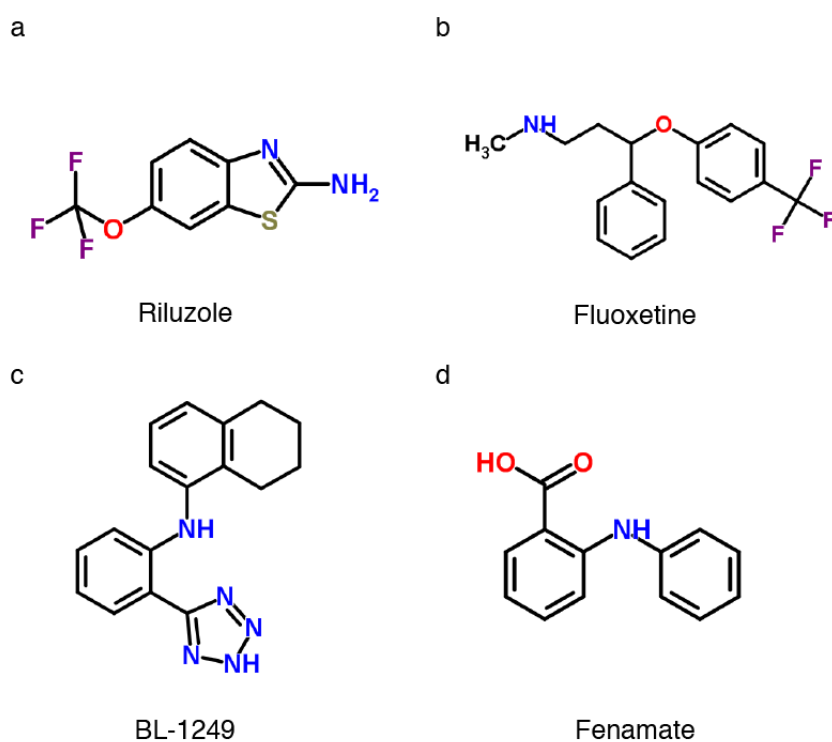


Figure 1.7: Pharmacological agents of K2P channels: (a) Riluzole activates TRAAK, but not TREK-1, (b) Fluoxetine (Prozac) inhibits TREK-1, (c) BL-1249 activates TREK-1, (d) Fenamate compound.

ways may activate or inhibit K2P channels depending on the K2P channel subtype. For example, the neuroprotective agent used in the treatment of amyotrophic lateral sclerosis, riluzole (Figure 1.7a), activates TRAAK, but not TREK-1 [76]. Although TREK-1 is transiently activated by riluzole, the inhibitory effect of protein kinase A (PKA)-phosphorylation of S333, following a rise in intracellular cyclic adenosine monophosphate (cAMP) levels [12], compensates for the activatory effect of riluzole. How riluzole increases intracellular cAMP levels is as yet unknown, but TRAAK, which is lacking the PKA-phosphorylation site of TREK-1, is permanently activated by riluzole [76]. Thus, an improved understanding of the interactions of K2P channels with secondary messengers such as highly charged anionic lipids may help to better understand the complex regulation and pharmacology of K2P channels.

Inhibition of TREK-1 by fluoxetine (Prozac) (Figure 1.7b) has been shown to induce dissociation of the C-terminal domain of TM4 (Figure 1.5) from the membrane [93], but progressive truncation of the C-terminal domain has not affected inhibition of TREK-1 by fluoxetine [109]. Moreover, TREK-1 and TRESK react oppositely upon binding of the putative potassium channel opener BL-1249 [(5,6,7,8-tetrahydronaphthalen-1-yl)-(2-(1H-tetrazol-5-yl)phenyl)amine] (Figure 1.7c). BL-1249 is related to the fenamate (Figure 1.7d) compound group of nonsteroidal anti-inflammatory drugs. While TREK-1 channel opening is promoted by BL-1249, TRESK channel activity is inhibited by BL-1249. These findings demonstrate the complexity of K2P channel pharmacology. The opposite pharmacology of TREK-1 and TRESK is likely due to the different topology of these K2P channels, and key to the understanding of the functional differences of these channels.

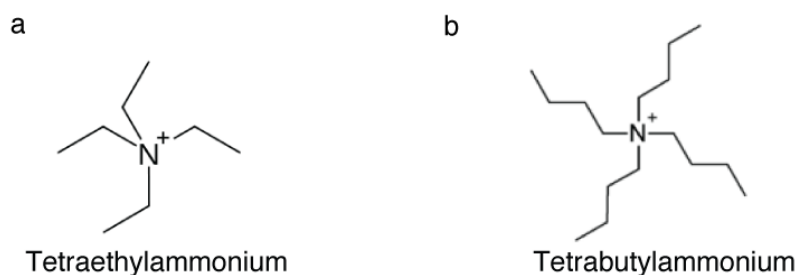


Figure 1.8: Quaternary ammonium ions. Tetraalkylammonium.

Quaternary ammonium (QA) ions are ion channel blockers commonly used to study K^+ channels (Figure 1.8). The inhibitory effect of tetraethylammonium (TEA) (Figure 1.8a) has long been exploited to probe the pore structure and gating mechanisms of K^+ channels [110]. Classically, K2P channels have been described as TEA-insensitive [111] and TEA has no inhibitory effect on TREK-1 when applied extracellularly [112]. However, if applied intracellularly, increasing the QA alkyl tail length leads to inhibition of TREK-1, TASK-3, and TRESK channels [34] (Figure 1.8b). Piechotta *et al.* further studied this intracellular QA sensitivity of TREK-1. They conducted a systematic cysteine mutagenesis scan of the TREK-1 channel pore to determine the residues that affect intracellular QA inhibition in TREK-1 [34]. Based upon this functional mutagenesis data, an improved TREK-1 homology model will be developed in Chapter 5. A TRESK model will also be derived, and these models are then employed to study K2P/lipid interactions.

1.3 Lipid interactions with K^+ channels

It has long been acknowledged that most ion channels are regulated by endogenous lipids [113, 114]. However, study of these interactions has been notoriously difficult [115]. Many methods exist that are indirectly up- or downregulating endogenous lipid concentrations, exploiting complex cellular pathways [11]. But, direct quantitative measurement of ion channel/lipid interactions is challenging except in cases where the lipid composition can be controlled, such as in reconstituted systems [116].

Most endogenous lipids are phospholipids derived from glycerol. These lipids have two fatty acid chains esterified to the first and second hydroxyl of a glycerol molecule, and differ by the head group, which is linked by a phosphodiester bond to the third hydroxyl carbon (Figure 1.9). The head group can either be a serine, ethanolamine, choline, or inositol group (Figure 1.9). The phospholipids phosphatidylserine (PS) and all phosphatidylinositols (PIs) are anionic, whereas phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are zwitterionic.

The lipid composition varies for different membranes throughout the cell [117]. A breakdown of the lipid composition of human dendritic cells is given by Subra *et. al.* [118] [43 % PC, 23 % PE, 12 % PI+PS, 9 % sphingolipids, and 13 % lysophospholipids (LPC)]. Anionic lipids, which are primarily found in the inner leaflet of the lipid bilayer, seem to be of particular importance, for all K^+ channels. Voltage-gated (Kv) potassium channel activity depends on anionic phospholipids [119, 120, 121]. Furthermore, anionic phospholipids directly bind to bacterial K^+ channels, such as the KcsA channel, which requires negatively

charged phospholipids to function [122, 123].

1.3.1 Kir channels and phosphoinositols

PIs may have negatively charged phosphate groups attached to the 3', 4' or 5' carbon of the inositol ring. The most important PI for K⁺ ion channel activation is the secondary messenger phosphatidylinositol-4,5-bisphosphate (PIP₂) (Figure 1.9b) [124]. Kir channels are sensitive to highly charged PIs [125, 126, 127]. The effect of PIP₂, which activates all Kir channel subtypes [128, 129], has been studied since 1996, after Fan *et al.* demonstrated the activatory effect of PIP₂ on cloned [130], and Hilgemann *et al.* on native K⁺ channels [131]. Systematic mutation of basic residues on the intracellular domain of Kir channels determined residues that affect PIP₂-sensitivity [50, 132, 133, 134]. A common co-regulatory role in Kir channel activation has been suggested for PIP₂ [135, 136, 137, 138, 139]. Kir channels also exhibit an absolute requirement for a secondary anionic lipid [140].

1.3.2 Sensitivity of TREK-1 to endogenous lipids

TREK-1 is activated by PIP₂ and inhibited by depletion of PIP₂ following Gq protein-coupled receptor (GqPCR)-mediated activation of phospholipase C (PLC). A polybasic motif in the TREK-1 C-terminus [141] links TREK-1 activation to association of the C-terminus with the cytosolic membrane leaflet [93]. A similar basic motif can be found in the mechanosensitive cationic large conductance (MscL) channel from *Escherichia coli* [142, 143]. Deleting the last 100 amino acids of TREK-1 retains PIP₂ sensitivity of TREK-1, whereas alanine replacement of the polybasic motif inhibits PIP₂ sensitivity [99]. However,

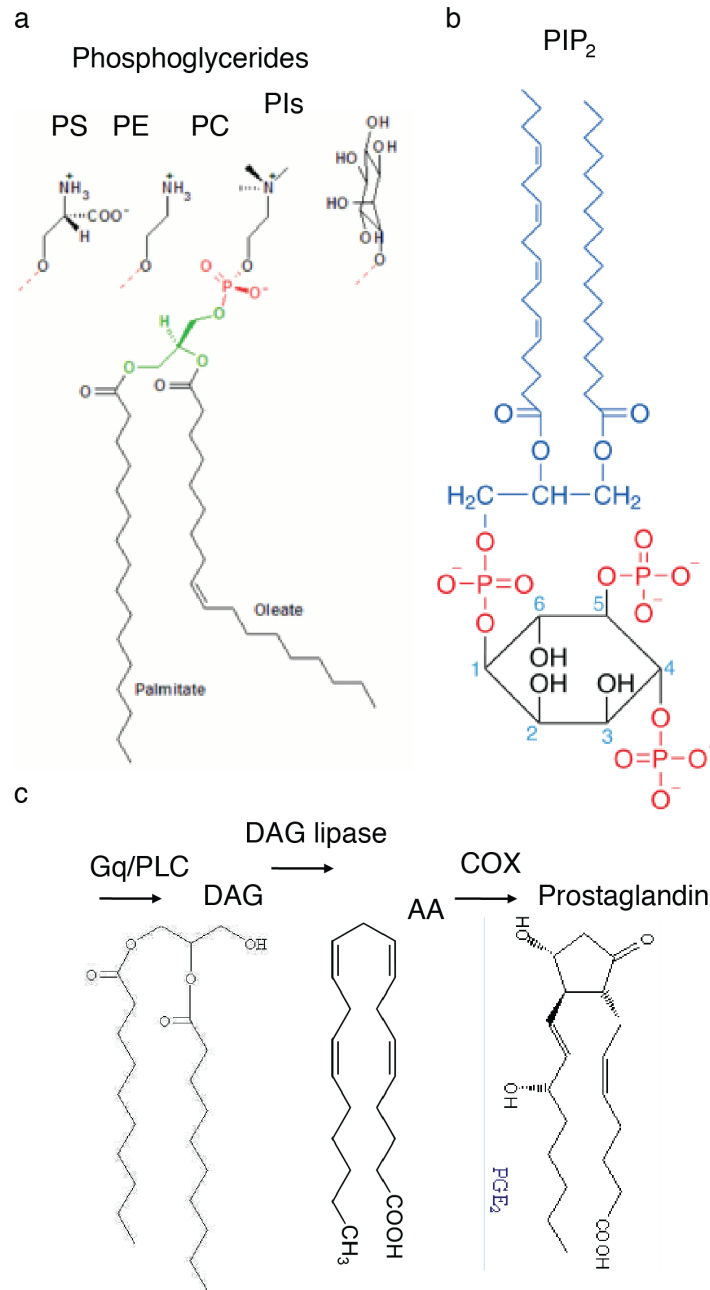


Figure 1.9: Endogenous lipids. **(a)** Phosphoglycerides PS (phosphatidylserine), PE (phosphatidylethanolamine), PC (phosphatidylcholine) and Pls (phosphatidylinositols). **(b)** The secondary messenger lipid PIP₂. **(c)** PIP₂ is metabolized into diacylglycerol (DAG), arachidonic acid (AA), and prostaglandin. From Wikimedia Commons, the free media repository © 2013

application of exogenous PIP₂ to TREK-1 has produced both activatory and inhibitory effects on TREK-1 channel activity [96]. The downstream effects of up- or downregulation of the endogenous PIP₂ concentration may complicate the study of PIP₂/K2P channel interactions [144]. For example, GqPCR stimulation activates PLC, hydrolysing PIP₂ to diacylglycerol (DAG) (Figure 1.9b), but there exist conflicting views, whether DAG directly inhibits TREK-1 [145, 99, 146].

Interestingly, TREK-1 is activated by arachidonic acid (AA) [147, 148], an ω -6 polyunsaturated fatty acid which is synthesized from DAG [149] (Figure 1.9b), and which plays an important role in neuroprotection [150]. Activation of TREK-1 channels following AA application leads to hyperpolarisation and reduced neuronal excitability. AA is also the precursor of the pro-inflammatory eicosanoid prostaglandin [151]. Moreover, membrane stretch and pressure opens TREK-1 and TRAAK channels [141, 152, 153]. However, K2P channels are not exclusively sensitive to endogenous lipids. The following section briefly outlines some interactions between non-endogenous lipids and TREK-1.

1.3.3 Sensitivity of TREK-1 to exogenous lipids

Ingestion of chilli pepper leads to well-known burning sensations. The underlying molecular mechanism involves excitation of somatosensory neurons through activation of transient receptor potential (TRP) channels, which respond to noxious heat [154]. However, unlike chilli pepper, ingestion of Szechuan pepper and other peppercorns from the *Zanthoxylum* genus leads to a unique tingling, numbing sensation [155, 156], caused by the alkylamide hydroxy- α -

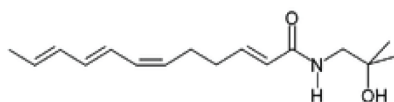
sanshool. This active compound from Szechuanese pepper differs from capsaicin, which is the active compound of chilli pepper from the *Capsicum* genus (Figure 1.10a).

It was suggested that capsaicin and sanshool act on a common set of ion channels to excite somatosensory neurons [157, 158]. However Bautista *et al.* reported inhibition of K2P channels TASK-1, TASK-3 and TREK by sanshool [159, 160]. Inhibition of K2P channels leads to increased neuronal excitability and is believed to be the reason for unique tingling parasthesia evoked by Szechuan pepper [161]. There is much interest in understanding the molecular and cellular mechanisms underlying the detection of chemical irritants. In order to investigate the molecular underpinnings of the tingling sensation provoked by sanshool and similar exogenous lipids, methods for the prediction of lipid/protein interactions, which are developed in this thesis, could prove useful.

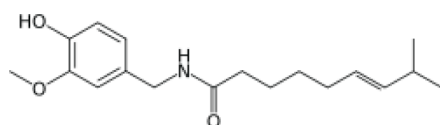
1.4 Thesis outline

This thesis describes computational studies on a variety of K^+ channels involved in regulating the electrical excitability of cells, building on previous work in the Sansom group [34, 162]. In particular, this thesis attempts to address three related questions.

First, can *in-silico* modelling and simulations describe the mechanism of gating in bacterial K^+ ion channels (Chapter 3)? Second, is it possible to extend this approach to use molecular modelling and simulation methods to obtain an open model of an eukaryotic K^+ channel, and are simulations able to predict



Hydroxy α sanshool
(Szechuan pepper, from plants of the
Xanthoxylum genus)



Capsaicin
(Chilli pepper, form plants of the *Capsicum* genus)

Figure 1.10: Exogenous lipds. The alkylamide hydroxy- α -sanshool and capsaicin.

the binding site of the activating ligand PIP₂ to a K⁺ channel (Chapter 4)? Third, can *in-silico* dockings combined with simulations predict PIP₂ interactions with a homology model of a K⁺ ion channel for which no crystal structure exists (Chapter 5)? Finally, in a drug discovery context, the ultimate aim would be to provide computational tools for the investigation of lipid/ion channel interactions, tools that may be used to study the interaction of endogenous and exogenous lipophilic molecules with K⁺ channel gating.

The thesis addresses these four related questions in three results chapters, and is organised as follows. Chapter 3 describes simulations of two X-ray crystal structures from the bacterial homolog of a eukaryotic Kir channel. The X-ray crystal structures are mutant structures determined in an apparently open state, and here, *in silico*-mutation of a residue at the helix bundle crossing combined with simulations will show that these structures are indeed in an open state.

A purely computational extension of the KirBac study is discussed in Chapter 4, in which the recent eukaryotic Kir2.2 channel structure serves as template to model the opening mechanism of Kir channels. Moreover, Chapter 4 focuses on PIP₂/Kir channel interactions and validates a method for the prediction of a PIP₂ binding site in a K⁺ channel.

Chapter 5 builds on the results of the PIP₂/Kir channel simulations in the previous chapter and considers the more complex case of a K2P channel (TREK-1) interacting with PIP₂. As there was no crystal structure of TREK-1 available at the time of writing, a homology model of TREK-1 had to be developed. A model of TRESK will also be developed.

Chapter 2

Theory and Methods

Simulations have long been used to study biomolecules in solution [163]. The study of ion channels, particularly the study of their mechanisms of gating, has benefited from combining computer simulations with mathematical models and experiments [164, 165], and molecular dynamics (MD) simulations increasingly contribute to the interpretation of functional data [166]. This chapter provides the theoretical background behind the computational methods used in this thesis to study K^+ channels in MD simulations and in continuum models. Finally, a brief description is given of the calculation of ligand binding sites in AutoDock [167].

2.1 Classical molecular dynamics

Classical MD simulations are well established, dating back to 1959 [168], and allow us to follow a microstate of a many-particle system in time. In classical MD, the laws that govern the time-evolution ($\{\mathbf{r}_i(t)\}, \{\mathbf{p}_i(t)\}$) of a particle

system, where $\{\mathbf{r}_i(t)\}$ refers to the position and $\{\mathbf{p}_i(t)\}$ to the momentum of the i -th particle¹ at time t , are deterministic, and the forces conservative, i.e. only depending on the positions and not on the velocities. Therefore, the phase space density $\rho(\{\mathbf{r}_i(t)\}, \{\mathbf{p}_i(t)\}, t)$ of a particle system of the Hamiltonian H is constant (Liouville's theorem² [171]):

$$\dot{\rho} = \frac{\partial \rho}{\partial t} + \{\rho, H\} = 0 \quad (2.1)$$

and the dynamics of ρ are formally equivalent to those of incompressible fluids [172]:

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{r}) = 0. \quad (2.2)$$

Next to classical MD simulations, methods that include stochasticity, such as Langevin mechanics or Brownian dynamics provide alternative ways to simulate biological systems [173]. In these methods, the solvent, which accounts for the majority of the computation time, is only implicitly taken into account to save computational time for calculating the protein dynamics [174]. Besides, *ab initio* calculations can be performed using quantum mechanical/molecular mechanics simulations (QM/MM) [175]. QM/MM simulations overcome some of the shortcomings of classical MD, such as lack of polarizability, but come at the expense of much higher computational cost [176, 177].

¹Vectors are printed in bold.

²Following Bohr [169], replacing the Poisson brackets in Eq. (2.1) with quantum mechanical operators (commutators), leads to the Heisenberg picture, which was shown to be equivalent to the Schrödinger formulation of quantum mechanics [170].

2.1.1 Averages and measures of variations

Statistical mechanics provide the tools to calculate expectation values of microscopic observables. To obtain macroscopic properties from simulations, microscopic properties need to be averaged. The ergodic hypothesis [178] states that the time average

$$\bar{A} = \frac{1}{\mathcal{T}} \int_0^{\mathcal{T}} A(t) dt \quad (2.3)$$

of a microscopic property A of an arbitrary microscopic N -particle state over the time $\mathcal{T}$ equals the expectation value¹ $\langle A \rangle$:

$$\langle A \rangle = \mathcal{Z}^{-1} \int \int A(\mathbf{r}, \mathbf{p}) \exp(-\beta H(\mathbf{r}, \mathbf{p})) d\mathbf{r}^{3N} d\mathbf{p}^{3N} \quad (2.4)$$

of that property, where N is the number of particles, obtained by averaging that property over an ensemble of microstates in phase-space:

$$\langle A \rangle = \bar{A}. \quad (2.5)$$

The time needed for time-averaged quantities to converge to the theoretical ensemble-average would be very long [179]. Nonetheless, simulations can provide valuable insights [180]. Firstly, MD simulations allow thought experiments, such as comparisons between simulations of wild-type and mutant protein structures. Secondly, coarse-grained (CG) approaches, in which a group of related particles is counted as one simplified, coarse particle, now enable access to long time scales in the μs to ms range, sufficient for the observation

¹ $\mathcal{Z} = \int \int \exp(-\beta H(\mathbf{r}, \mathbf{p})) d\mathbf{r}^{3N} d\mathbf{p}^{3N}$ is the partition sum in the canonical ensemble, and $\beta = 1/(k_B T)$, where k_B is the Boltzmann constant, and T the temperature.

of lipid/protein interactions [181].

How can properties of MD simulations then be measured in practice? The property A could be a point $\mathbf{r}$ in space, such as the position of the C_α -atom of a protein residue or the geometric centre of a lipid head group. The root mean square fluctuation (RMSF) of $\mathbf{r}$, is then a measure of the variation of $\mathbf{r}$ in space, and defined using Eq. (2.6):

$$RMSF = \sqrt{\overline{(r(t) - \bar{r})^2}} = \sqrt{\frac{1}{\mathcal{T}} \sum_{t=1}^{\mathcal{T}} (r(t) - \bar{r})^2} \quad (2.6)$$

The RMSF is a measure of the variation of $\mathbf{r}$. On the other hand, the root mean square deviation (RMSD) describes the drift of $\mathbf{r}$ with respect to a reference value $\mathbf{r}(t_0)$ at time t_0 . Here, $\mathbf{r}(t_0)$ could be the starting structure of an ion channel, and the RMSD would be a measure for its conformational stability.

$$RMSD = \sqrt{\langle (\mathbf{r}(t) - \mathbf{r}(t_0))^2 \rangle} = \sqrt{\frac{1}{N} \sum_{i=1}^N (\mathbf{r}_i(t) - \mathbf{r}_i(t_0))^2}, \quad (2.7)$$

where the sum is taken over all vectors $\{\mathbf{r}_i\}$ at times t , the protein structure at time t , and over all reference vectors $\{\mathbf{r}_i(t_0)\}$ at time t_0 (corresponding to the starting structure at time t_0).

In similar ways, other properties, such as the radius of gyration of the protein, or the centre-of-mass distance between a group of atoms, can be calculated [182].

Having reviewed the fundamentals of classical MD simulations, the next section will outline what ‘classical’ means in practice.

2.1.2 Born-Oppenheimer approximation

Classical MD simulations treat each atom as a particle with a point charge located at its centre. Following Born and Oppenheimer [183], as the mass of the nuclei is much larger than the electron mass, the electrons may be considered to follow instantaneously the movement of the nuclei. The wave function ϕ of the electron can therefore be separated from the wave function Φ of the nuclei using the following *ansatz* for the wave function Ψ of the whole atom:

$$\Psi(\mathbf{R}, \mathbf{r}_{\text{el}}) = \phi(\mathbf{r}_{\text{el}}|\mathbf{R})\Phi(\mathbf{R}) \quad (2.8)$$

Here, $\mathbf{R}$ stands for the position of the nuclei and $\mathbf{r}_{\text{el}}$ for the position of the electrons of the atom. Together with the stationary Schrödinger equation, $H\Psi = E\Psi$, this yields the Born Oppenheimer equation and an effective potential $V(\Phi)$ that only depends on the centres of the atom nuclei, but not on the electron positions. This potential $V(\Phi)$, together with the classical Hamiltonian

$$H = \frac{\mathbf{p}^2}{2m} + V(\Phi) \quad (2.9)$$

describes all aspects of the now classical atom with mass m and it follows the Newtonian formulation of the Hamiltonian system,

$$m\ddot{\mathbf{r}} = \mathbf{F}, \quad (2.10)$$

where the gradient of the Born-Oppenheimer energy surface yields the force $\mathbf{F}$ acting on the atom:

$$\mathbf{F} = -\nabla V. \quad (2.11)$$

Newton's equations provide a set of $3N$ second order coupled differential equations, where N is the number of particles, which can be solved using the Groningen Machine for Chemical Simulation package (GROMACS) [184, 185], a molecular simulations software used throughout this thesis. In order to be able to calculate the forces $\mathbf{F}$ on each particle, GROMACS provides the functional form of the effective Born-Oppenheimer potential V , which determines the general shape of the potential, and is given the parameters, which determine the specific interaction strength for each atom type [186]. The functional form of the potential will be discussed next, following the package's documentation [187] and other text books on the subject [188, 189]. This will clarify the meaning of the 'parameters' – they turn out to be the *force field*.

2.1.3 Force fields for biomolecular simulations

The many-body potential $V\{\mathbf{r}_i\}$, where the index i refers to an individual particle, is approximated in GROMACS by the sum of the pairwise additive interactions between non-bonded and selected bonded interactions:

$$V = V_{nb} + V_b, \quad (2.12)$$

where the non-bonded interactions V_{nb} include contributions of the electrostatic (Coulomb) V_C and van der Waals (Lennard-Jones) V_{LJ} potential:

$$V_{nb}(\{\mathbf{r}_i\}) = \sum_{i < j} V_C(\mathbf{r}_i, \mathbf{r}_j) + \sum_{i < j} V_{LJ}(\mathbf{r}_i, \mathbf{r}_j). \quad (2.13)$$

Bonded interactions are calculated using two-, three-, or four-atom poten-

tials for bond stretch, angular, and dihedral vibrations:

$$V_b(\{\mathbf{r}_i\}) = \sum_{(i,j)} V_{bond}(\mathbf{r}_i, \mathbf{r}_j) + \sum_{(i,j,k)} V_{angle}(\mathbf{r}_i, \mathbf{r}_j, \mathbf{r}_k) + \sum_{(i,j,k,l)} V_{torsion}(\mathbf{r}_i, \mathbf{r}_j, \mathbf{r}_k, \mathbf{r}_l) \quad (2.14)$$

Here, the first sum V_{bond} restrains the covalent bond length, the second sum V_{angle} the angle between two bonds, and the third sum $V_{torsion}$ the proper and improper dihedral angles. Pairwise potentials are evaluated at distances $r_{ij} = |\mathbf{r}_i - \mathbf{r}_j|$.

Non-bonded interactions

The Lennard-Jones potential V_{LJ} determines the non-bonded interactions between neutral atoms and is given by:

$$V_{LJ}(r_{ij}) = 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] \quad (2.15)$$

where ϵ_{ij} indicates the depth of the potential well and σ_{ij} the distance at which the potential energy is zero for the pairwise potential between the i -th and j -th particle. The parameters are dependent on the force field the atom types. The Lennard-Jones potential can also be written in the following form:

$$V_{LJ}(r_{ij}) = \frac{C_{ij}^{(12)}}{r_{ij}^{12}} - \frac{C_{ij}^{(6)}}{r_{ij}^6}, \quad (2.16)$$

The coefficients $C_{ij}^{(12)}$ and $C_{ij}^{(6)}$ depend on the atom types of the atom pair i, j . An alternative potential for long-ranged interactions is the Buckingham poten-

tial, which substitutes the empirical $1/r^{12}$ by a functional of the overlap integral of the wave function of the interacting atoms. The first, repulsive term in the Lennard Jones potential comes from the the quantum mechanical Pauli exclusion principle, which prohibits two valence electrons to occupy the same state. The second term is the electrostatic dipole-dipole interaction, which follows for charged particles from a multipole expansion [190]. For uncharged particles, the second term represents the quantum mechanical London force between two uncharged atoms [191].

Long-ranged electrostatic interactions between charged atoms are calculated from Coulomb's law:

$$V_C(r_{ij}) = \frac{1}{4\pi\epsilon_0} \frac{q_i q_j}{r_{ij}}, \quad (2.17)$$

where ϵ_0 is the dielectric constant, and q_i and q_j are the (partial) charges of two atoms at distance r_{ij} . The assignment of partial charges is an important step in the parameterisation of a force field.

Bonded interactions

The interaction between bonded atoms are for small deviations from the equilibrium similar to the behaviour of a harmonic oscillator. The bond-stretch potential therefore follows Hooke's law

$$V_{bond}(r_{ij}) = \frac{k_{ij}^b}{2} (r_{ij} - b_{ij})^2, \quad (2.18)$$

where the equilibrium bond length b_{ij} and the force constant k_{ij}^b depend on the types of interacting atoms. An alternative, and equally valid, representation is

the fourth power potential:

$$V_{bond}(r_{ij}) = \frac{\tilde{k}_{ij}^b}{4}(r_{ij}^2 - b_{ij}^2)^2. \quad (2.19)$$

The bond angle vibrations are described using a harmonic potential on the angle θ_{ijk} given a triplet of atoms i, j, k :

$$V_{angle}(\theta_{ijk}) = \frac{k_{ijk}^\theta}{2}(\theta_{ijk} - \theta_{0,ijk})^2, \quad (2.20)$$

where $\theta_{0,ijk}$ is the bond angle at equilibrium, and k_{ijk}^θ the force constant. Alternatively, a simplified version may be used to calculate angle vibrations:

$$V_{angle}(\theta_{ijk}) = \frac{k_{ijk}^\theta}{2}(\cos \theta_{ijk} - \cos \theta_{0,ijk})^2. \quad (2.21)$$

Bond torsions are calculated using a periodic potential on the proper dihedral angle ϕ_{ijkl} , where ϕ_{ijkl} is the angle between the two normal vectors of planes i, j, k and j, k, l :

$$V_{dihedral}(\phi_{ijkl}) = k_{ijkl}^\phi(1 + \cos(n\phi_{ijkl} - \phi_{0,ijkl})), \quad (2.22)$$

with multiplicity n , phase shift $\phi_{0,ijkl}$, and dihedral force constant k_{ijkl}^ϕ .

Dihedral angles are defined according to the IUPAC/IUB convention, such that the *cis* configuration (i and l on the same side) corresponds to zero. Alternatively, the Ryckaert-Bellemans potential can be used:

$$V_{dihedral}(\phi_{ijkl}) = \sum_{n=0}^5 C_n \cos^n(\phi_{ijkl} - \pi), \quad (2.23)$$

and some force fields use the first four terms of a Fourier series in the dihedral potential, a form similar to the Ryckaert-Bellemans potential.

An additional dihedral potential is used to keep improper dihedral angles in plane and to avoid inversion of tetrahedral groups. Here, ξ_{ijkl} denotes the improper dihedral angle, $\xi_{0,ijkl}$ the improper dihedral equilibrium angle, and k_{ijkl}^{ξ} the force constant of the improper dihedral potential:

$$V_{improper}(\xi_{ijkl}) = \frac{k_{ijkl}^{\xi}}{2} (\xi_{ijkl} - \xi_{0,ijkl})^2 \quad (2.24)$$

Next, the choice of the force field which parameterises the above equations is discussed.

Choice of force field

In biomolecular simulations, force fields define interactions between atoms by assigning atom type-specific parameters to the coefficients of the equations Eqs. (2.15) – (2.24). These coefficients are obtained through comparison of MD simulations with thermodynamic and chemical properties gained from experiments (e.g. water-octanol partitioning) or quantum mechanical calculations (e.g. partial charges). Several classical force fields for simulations of proteins exist today, such as the Chemistry at Harvard Molecular Mechanics (CHARMM) force field [192], the Groningen Molecular Simulation (GROMOS) force field [186], the Assisted Model Building with Energy Refinement (AMBER) [193] and the Optimised Potentials for Liquid Simulations (OPLS) [194]. Their parameterisation has been a compromise between computational efficiency and accuracy [195].

The choice of a force field depends on the requirements and needs to be

addressed. For the PIP_2/K^+ channel interactions studied in this thesis, the GROMOS force field and the lipid parameters from Berger and co-workers were chosen, as this combination is compatible with parameters for PIP_2 [162], and has been used previously to study lipid/ion channel interactions [162]. Furthermore, as a united-atom force field, GROMOS is computationally efficient. It removes high-frequency vibrations by incorporating non-polar hydrogen atoms into larger united atoms. This allows to use a larger time step. In this thesis, atomistic simulations are therefore carried out with the GROMOS96, version 43a1, force field and the lipid parameter set from Berger and co-workers [196, 186]. Coarse-grained simulations are performed using the Martini force field [197], as described in Section 2.2. The Single Point Charge (SPC) water model [198], is employed in all simulations.

In the GROMOS force field, polar hydrogen atoms and hydrogen atoms on rings are treated explicitly, although their bond length and bond angle are constrained. The SPC water model is a non-polarisable three-site model, with a partial negative charge at the oxygen, balanced by corresponding positive charges on the hydrogen atoms [198]. To compensate for the non-polarisability of SPC water, the dipole of SPC is set at a larger value than its corresponding experimental value. GROMOS uses the fourth-power potential, Eq. (2.16), for bonds lengths, Eq. (2.21) for bond angles and Eq. (2.22) for proper dihedrals.

2.1.4 Algorithms

Newton's equations of motion for the potential $V = V_{nb} + V_b$ can not be solved analytically. Thus, several algorithms have been developed to compute the time-evolution of the microstate $(\{\mathbf{r}_i(t)\}, \{\mathbf{p}_i(t)\})$ numerically.

Integration of the equation of motions

The equations of motion are integrated using the derivatives of the positions at discrete time steps Δt . In the GROMACS simulation suite, there exist several integration schemes, which different properties regarding computational efficiency, stability, and energy conversation. In this thesis, the Leap-Frog integrator, a variant of the Verlet algorithm [199], is employed. The sum of the forward and backward taylor expansions of the coordinates $\mathbf{r}(t)$ in time,

$$\begin{aligned}\mathbf{r}(t + \Delta t) &= \mathbf{r}(t) + \dot{\mathbf{r}}(t)\Delta t + \ddot{\mathbf{r}}(t)\frac{\Delta t^2}{2} + \mathcal{O}(\Delta t^3) \\ \mathbf{r}(t - \Delta t) &= \mathbf{r}(t) - \dot{\mathbf{r}}(t)\Delta t + \ddot{\mathbf{r}}(t)\frac{(\Delta t)^2}{2} - \mathcal{O}(\Delta t^3)\end{aligned}\tag{2.25}$$

gives the Verlet algorithm, which updates the positions according to:

$$\mathbf{r}(t + \Delta t) \approx 2\mathbf{r}(t) - \mathbf{r}(t - \Delta t) + \ddot{\mathbf{r}}\Delta t,\tag{2.26}$$

where $\ddot{\mathbf{r}} = \mathbf{F}/m$ and the error is in the order of $\mathcal{O}(\Delta t^4)$. It can be shown that the Verlet is symmetric under time-reversal and that the phase space density $\rho(\{\mathbf{r}_i(t)\}, \{\mathbf{p}_i(t)\})$ is conserved. These integration schemes solve the equations of motions of classical Hamiltonian systems with global stability [187, 189]. The leap-frog integrator, which shares these properties, is used throughout this thesis:

$$\begin{aligned}\mathbf{r}(t + \Delta t) &\approx \mathbf{r}(t) + \dot{\mathbf{r}}(t + \frac{1}{2}\Delta t)\Delta t \\ \dot{\mathbf{r}}(t + \frac{1}{2}\Delta t) &\approx \dot{\mathbf{r}}(t - \frac{1}{2}\Delta t) + \ddot{\mathbf{r}}(t)\Delta t.\end{aligned}\tag{2.27}$$

Time step and constraints

Positions and velocities were updated every $\Delta t = 2$ fs. In general, the time step should be one order of magnitude smaller than the period of time of the fastest motion [188]. The period of the C-H, O-H, and N-H bond oscillations is approximately 10 fs [187]. In MD simulations, the bond lengths of bonded interactions can be constrained using the linear constraint solver (LINCS) [200] or SHAKE algorithm [201]. For the simulations reported in this thesis, bond lengths were reset to their reference length, after each time step using the LINCS algorithm. Bond angles were unconstrained. For SPC water, the optimized water constraint algorithm SETTLE was used [202].

Position restraints

GROMACS provides the functionality to restrain the protein's heavy atoms to fixed reference positions to avoid instabilities at the beginning of the simulation. The force is derived from a harmonic restraint potential

$$V_{pr}(\mathbf{r}) = \frac{k_{pr}}{2} |\mathbf{r} - \mathbf{r}_0|^2\tag{2.28}$$

with force constant k_{pr} and reference position $\mathbf{r}_0$. In each atomistic simulation, the heavy atoms of the protein were restrained for the first 1 ns to allow the

equilibration of solvent and lipids.

Periodic boundary conditions

As all atoms are confined to a simulation box of limited dimensions l , periodic boundary conditions are used. Each time an atom reaches the box walls, its image enters the box on the opposite side. Every particle thus interacts with imaginary neighbours, the periodic images, of particles, which are more than $l/2$ of the box length away, if l is the length of the box size. Hence, to avoid artefactual summation of long-range dispersive van der Waals interactions over an infinite number of periodic images, the vdW cut-off was set to less than half the box size. The cut-off for long-range electrostatic forces is treated with by the Particle Mesh Ewald summation method [203, 204, 205].

Temperature and pressure coupling

Integration of the equations of motion for a sufficiently long time returns an ensemble of microstates ($\{\mathbf{r}_i(t)\}, \{\mathbf{p}_i(t)\}$). Expectation values of macroscopic variables, and expressions of thermodynamic quantities, such as compressibility and heat capacity can then be derived from the partition sum $\mathcal{Z}$ of the ensemble [178]. The form of the $\mathcal{Z}$, and thus the derivation of macroscopic variables depends on the variables which are conserved:

In the microcanonical ensemble (N, V, E), which describes an isolated system, the number of atoms N , the volume V , and the energy E are fixed. In the canonical ensemble (N, V, T), the system is kept in contact with a thermostat at temperature T , the number of atoms N , and the volume V are fixed. The energy E is no longer conserved. A system in the isobaric-isothermal ensemble ($N, P,$

T) is in thermal balance with a heat bath at temperature T , where the pressure P and atom number N are fixed. It exchanges volume (and work) with the barostat. The grand-canonical ensemble (μ, V, T) , describes an open system in contact with a particle reservoir fixing the energy μ required to add an atom to the system. While the volume and the temperature remain fixed, the number of atoms can vary. This describes an open system.

Molecular dynamics simulations are carried out in the NPT ensemble, as most biological processes occur at constant pressure and temperature. To ensure that the temperature remains constant, GROMACS provides the Berendsen thermostat, which uses a weak coupling scheme to exponentially relax the temperature to the reference temperature [206, 207]. Although weak coupling schemes do not strictly lead to a thermodynamic ensemble, they are easy to implement. A class of thermostats called extended Hamiltonian methods, such as the Nosé-Hover thermostat, generates thermodynamic ensembles that are well defined, but these are conceptually more complex and produce an oscillatory relaxation to the reference temperature. The simulations in this thesis were performed using the Berendsen thermostat with a slow relaxation time of 0.1 ps ($\Delta t = 2$ fs). The temperature T is calculated from the velocities with the help of the equipartition theorem:

$$\frac{1}{2}\nu k_B T = E_{kin} = \frac{1}{2} \sum_i m_i \dot{r}_i^2 \quad (2.29)$$

where k_B is the Boltzmann constant, and ν the number of degrees of freedom

of a system with N atoms of mass m_i , velocity $\dot{r}_i$ and N_c constraints:

$$\nu = 3N - N_c - N_{com} \quad (2.30)$$

The number $N_{com} = 3$ needs to be subtracted to account for the movement of the center-of-mass of the system. The deviations in temperature are adjusted according to the Berendsen thermostat:

$$\frac{dT}{dt} = \frac{T_0 - T}{\tau} \quad (2.31)$$

Deviations from the reference temperature T_0 will relax with the time constant τ . To adjust the temperature, velocities are rescaled by factor λ :

$$\lambda = \sqrt{\left[1 + \frac{\Delta t}{\tau_T} \left\{ \frac{T_0}{T(t - \frac{1}{2}\Delta t)} - 1 \right\}\right]} \quad (2.32)$$

where the velocity rescaling τ_T is related to the temperature rescaling τ by

$$\tau = \frac{2C_v\tau_T}{\nu k_B}. \quad (2.33)$$

C_v is the heat capacity, ν the number of degrees of freedom, and k_B the Boltzmann constant. The change in kinetic energy from scaling the velocities is distributed between the kinetic and potential energy, which is why $\tau \neq \tau_T$.

The pressure is controlled using the Berendsen barostat (in coarse-grained simulations) or the Parrinello-Rahman barostat [208]. The Berendsen barostat involves calculation of the force virial and the pressure tensor, in order to determine the rescaling factor of the particle positions and cell dimensions. Using

a weak coupling scheme, the pressure is relaxed in the Berendsen barostat exponentially [206]:

$$\frac{dP}{dt} = \frac{P_0 - P}{\tau_p} \quad (2.34)$$

P_0 is the reference pressure and τ_p the pressure coupling constant. The scaling matrix μ_{ij} which adjusts box vectors and atom coordinates at each time step is given by:

$$\mu_{ij} = \delta_{ij} - \frac{\Delta t}{3\tau_p} \beta_{ij} \{P_{0,ij} - P_{ij}(t)\}. \quad (2.35)$$

where δ_{ij} is the Kronecker-Delta symbol and β_{ij} the compressibility tensor. In all simulations, the compressibility β was semi-isotropically set to zero in the z-direction, scaling only with $\beta_{xx} = \beta_{yy} = 3.0 \times 10^{-4} \text{ bar}^{-1}$. and in the x-y plane of the membrane. The pressure was fixed at 1.0 bar with a slow relaxation time of $\tau_p = 1.0 \text{ ps}$.

Although the Berendsen barostat corrects pressure deviations, it does not yield a well-defined thermodynamical NPT ensemble. The Parrinello-Rahman pressure coupling was therefore used for atomistic simulations, with $\beta = 4.5 \times 10^{-5} \text{ bar}^{-1}$. The Parrinello-Rahman barostat is discussed in detail by Nosé and Klein [209].

2.2 Coarse grain simulations

Molecular dynamics simulations of lipid-protein systems have led to important insights into cellular function. In small confined systems, such as the selectiv-

ity filter of K^+ channels, atomistic simulations have contributed greatly to the study of protein structure and function. However, many cellular processes occur on time scales longer than those that can be simulated in classical MD simulations, despite the continuing progress in computer hardware and software development [210]. Next to continuum approaches, a range of coarse-graining (CG) methods has been developed in which each atom is mapped on typically two to five CG beads [211, 212, 197]. This reduces the number of particles and the number of degrees of freedom, and smoothens the energy landscape. Therefore, CG methods can be used with time steps which between 10 fs to 40 fs, more than a magnitude of order larger than the time steps in many atomistic simulations (ca. 2 fs). Furthermore, the sampling of the energy landscape is up to four times faster. Thus CG simulations lead to an increase in the attainable time and length scales.

2.2.1 Martini force field

The Martini force field was introduced by Marrink *et al.* [213], based on a CG force field for the simulation of lipids [214]. It was later extended by Monticelli *et al.* to include proteins (Martini version 2.1) [197]. Throughout this thesis, the Martini force field version 2.1 was used, described below, following [213, 197].

Particles types

The Martini force field uses a four-to-one mapping of atomistic to CG particles, with the exception of aromatic residues, which are mapped with a two-to-one or three-to-one scheme. The model includes four main types of particles, polar (P), apolar (C), nonpolar (N), and charged (Q). Polar particles are typically

used for hydrophilic groups of atoms that have partial charges, while apolar atoms are used for neutral and hydrophobic groups of atoms. Non-polar particles are intermediates between polar and apolar particles. Charged particles are representing groups of atoms with a full charge. Each particle type is further distinguished by its hydrogen capabilities (d = donor, a = acceptor, da = donor/acceptor, 0 = none) or by its degree of polarity (ranging from 1 = lower polarity to 5 = higher polarity). Table 2.1 lists the mapping between all amino acids and CG particles. The CG parameters for PIP₂ are given in Chapter 4, Figure 4.4.

The side chains of apolar amino acids Leu, Pro, Ile, Val, Cys and Met are modelled as C particle, while the side chains of polar uncharged amino acids Thr, Ser, Asn and Gln are represented by a P particle. Small negatively charged side chains Glu and Asp are modelled as a Q particle, and side chains of large charged residues are made of two distinct particles. A special class of ring particles represents aromatic residues. Protein backbone particle types are set depending on the secondary structure. The more flexible, the more polar the CG particle type. In coils or bends, the backbone is assigned a P particle. Backbone particles in helical or sheet-like structures, which are fairly rigid due to inter-backbone hydrogen networks, are modelled as less polar N-type particles. For reasons of computational efficiency, the mass of each particle type is set to 72 Da.

Particle interactions

Non-bonded interactions are described by Lennard-Jones or Coulomb potentials, Eqs. (2.15) and (2.17). The Coloumb interactions between charged (Q)

Table 2.1: Amino acid side chains and coarse grain (CG) particle types.

Side chain	CG particle
Leu	C1
Ile	C1
Val	C2
Pro	C2
Met	C5
Cys	C5
Ser	P1
Thr	P1
Asn	P5
Gln	P4
Asp	Qa or P3 (uncharged)
Glu	Qa or P1 (uncharged)
Arg	N0-Qd or N0-P4 (uncharged)
Lys	C3-Qd or C3-P1 (uncharged)
His	SC4-SP1-SP1
Phe	SC4-SC4-SC4
Tyr	SC4-SC4-SP1
Trp	SC4-SP1-SC4-SC4

particles are effectively screened by setting the permittivity to $\epsilon = 15$. The well-depth of the pair-wise Lennard-Jones potentials depends on the type of interacting CG particles and ranges from 2.0 kJ mol^{-1} to 5.6 kJ mol^{-1} . The effective van der Waals size of particles, modelled by the parameter $\sigma = 4.7 \text{ \AA}$, except for a few cases, such as ring-ring interactions, which are modelled at $\sigma = 4.3 \text{ \AA}$. Coulombic interactions are smoothly cut off at 1.2 nm . Lennard-Jones interactions are shifted with a twin-range cut-off such that long-range interactions of uncharged particles smoothly vanish between 0.9 nm to 1.2 nm .

Bonded interactions are modelled using harmonic potentials, as described above, Eqs. (2.18, 2.21, 2.24), where proper dihedrals restraints are calculated according to the periodic trigonometric potential, Eq. (2.22). Backbone-backbone bond lengths are all set to $3.5 \text{ \AA}$. Their force constants, angles and dihedrals depend on the secondary structure, not on the amino acid type. Backbone-side chain bond lengths and angles are independent of the secondary structure, but vary with the amino acid type. Dihedral restraints are imposed on helical regions of the backbone. It is important to note that CG force fields can therefore not be used to predict changes in secondary structure, which is why atomistic simulations are used to verify CG predictions in this study. In order to constrain secondary and tertiary structure, an elastic network model can also be used with the Martini force field [215].

Parameterization

Parameterization of the Martini 2.1 force field is based on reproducing the partitioning free energies ΔG of chemical compounds between polar and apolar phases of chemical compounds. The particle types are assigned to maximise

the agreement between the predicted densities of amino acids in water ρ_{water} and oil phases ρ_{oil} :

$$\Delta G = k_B T \ln \frac{\rho_{oil}}{\rho_{water}}, \quad (2.36)$$

with experimental cyclohexane/water partitioning data of amino acid analogues at room temperature [216, 217]. In some cases, to optimise the parameters, Monticelli *et al.* calculated the potential of mean force for translocation of the amino acid between water and a lipid bilayer model membrane [197]. Parameters for bonded interactions (bond lengths, bond angles, dihedral angles, and their respective force constants) are derived from structures of the Protein Data Bank. Parameters for non-bonded interactions are the same for amino acids as for lipids [213].

2.2.2 Multi-scale approach

Although the Martini force field has been used previously to study K^+ channels [218], coarse grain simulations come short to the level of detail that enables comparison with high-resolution X-ray crystal structures and need to be carefully interpreted when studying large conformational changes in membrane proteins. In Chapter 4, where CG simulations are used to predict K^+ channel/lipid interactions, the CG simulation is therefore converted into an atomistic representation for further simulation. This so-called multi-scale approach has been developed to study protein/lipid interactions of proteins in lipid bilayers [219], and has been applied widely to membrane/protein systems [220, 221, 222]. It is also used to self-assemble membrane proteins in a lipid bilayer. Other multi-

scale approaches couple atomistic force fields with CG representations in a hybrid scheme, using virtual sites [223].

A multi-scale method developed by the Sansom group *et al* is used throughout this thesis [162]. In a fragment-based reconstruction procedure, CG lipids are aligned with atomistic lipid fragments from a purpose-built library. The atomistic structure of the protein can be rebuilt using Pulchra [224] and missing side chains can be modelled using MODELLER [225]. In this thesis, only the lipids are reconstructed from CG. The CG protein is converted into an atomistic representation by alignment with an X-ray crystal structure. Alternatively, using a method by Rzepiala *et al.*, atomistic protein structures can also be reconstructed from CG using short MD simulations and dihedral restraints [226].

All MD simulations were carried out in GROMACS [185]. Specific parameters can be found in the ‘Methods’ section of each chapter of this thesis.

2.3 Continuum electrostatics

Continuum electrostatics describe the electrostatic potential in the environment of a charged protein in solution. The protein ($\epsilon_p \approx 10$) is described by a set of fixed charges ρ_f , whereas the solution is described by a continuum ($\epsilon_w \approx 78$) in which a set of mobile charges ρ_m is distributed. Debye-Hückel (1923) [227], based on the work of Gouy (1910) [228] and Chapman (1913) [229], derived the Poisson-Boltzmann equation, which can be used to calculate the electrostatic potential $V(\mathbf{r})$. The distribution of mobile ions in a solvent follows Maxwell-

Boltzmann statistics, to maximise the entropy of mixing [230]:

$$\rho_m(\mathbf{r}) \propto \exp\left(\frac{-Z_i q V(\mathbf{r})}{k_B T}\right), \quad (2.37)$$

where $V(\mathbf{r})$ is the electrostatic potential at point $\mathbf{r}$, q the proton charge, and Z_i the valency of the i -th ion species, k_B Boltzmann's constant, and T the temperature. The fixed charges of the protein are given by

$$\rho_f(\mathbf{r}) = \sum_i q_i \delta(\mathbf{r} - \mathbf{r}_i), \quad (2.38)$$

where q_i is the charge of atom i . Inserting the total charge $\rho_{tot} := \rho_f + \rho_m$ into Poisson's equation

$$\nabla \cdot [\epsilon(\mathbf{r}) \nabla V(\mathbf{r})] = -4\pi \rho_{tot}(\mathbf{r}) \quad (2.39)$$

in which $\epsilon(\mathbf{r})$ is the position-dependent dielectric constant, yields Poisson-Boltzmann equation (in Gaussian units):

$$\nabla \cdot [\epsilon(\mathbf{r}) \nabla V(\mathbf{r})] = -4\pi \rho_f(\mathbf{r}) - 4\pi \sum_i c_i^\infty Z_i q \exp\left(\frac{-Z_i q V(\mathbf{r})}{k_B T}\right) \chi(\mathbf{r}) \quad (2.40)$$

Here, the first term on the right hand side describes the fixed protein charges, while the second term describes the charge distribution of mobile ion species with valency Z_i , bulk density c_i^∞ (distance far away from the protein), and $\chi(\mathbf{r})$ prohibits the mobile ion positions to overlap with the fixed protein charged positions, it is 1 in the solvent and 0 in the protein. Similarly, $\epsilon(\mathbf{r}) = \epsilon_w$ in the solvent and $\epsilon(\mathbf{r}) = \epsilon_p$ in the protein.

The electrostatic energy required to charge the protein (the fixed charges) in

the presence of the mobile ions is [231]:

$$\Delta G_{el}^P = \frac{1}{2} \int \rho_f(\mathbf{r}) V(\mathbf{r}) d\mathbf{r} \quad (2.41)$$

The electrostatic energy of binding $\Delta G_{el}(z)$ of an ion to a position z along the pore axis of a protein (K^+) channel can be calculated:

$$\Delta G_{el}(z) = \Delta G_{el}^{P-I} - \Delta G_{el}^I - \Delta G_{el}^P. \quad (2.42)$$

ΔG_{el}^{P-I} is the electrostatic energy of the protein-ion complex, with the ion bound to position z on the ion channel pore axis. ΔG_{el}^I denotes the electrostatic energy of the ion in the bulk, and ΔG_{el}^P is the total electrostatic self-energy of the protein in solvent as defined above. The ion is modelled as a sphere with the dielectric of the protein and with a Born radius (2.2 Å) that matches experimental values [232].

The Poisson-Boltzmann equation can be solved with finite-difference or finite-elements methods, by discretisation on a grid and (fast) Fourier transform [233, 234]. The Advanced Poisson Boltzmann Solver (APBS) software [235] was used in Chapter 3 and 4 of this thesis, together with the Bornprofiler scripts from the Sansom group.

2.4 Homology modelling

Homology modelling predicts proteins structures from similar or homologous, protein structures [236]. The software MODELLER is used in this thesis to model structures of K2P channels [225, 237]. From a sequence alignment of

the K2P target sequence with one or more template sequences, a set of spatial constraints is derived, which are included in an energy function parameterised in CHARMM. The output model is optimised by minimisation of the energy function using a biconjugate gradient method [238]. Short MD simulations, in which the temperature is first raised and then slowly cooled minimise the risk of trapping in a steep and narrow energy minima ('simulated annealing') [236]. Other methods that have been developed to model membrane proteins are MEDELLER [239], or methods based on the covariance of mutations in large sequence alignments (PSICOV) [240].

2.5 Docking calculations

In-silico docking plays an important role in the discovery of new drugs and in the study of protein-protein interactions. Molecular docking techniques have been optimized for high-throughput requirements encountered in the pharmaceutical drug discovery. Large databases of – typically proprietary – small molecules are tested against a 'target' protein. Through this 'virtual screening' process, promising 'lead' compounds can be identified and, further developed using pharmacophore approaches, quantitative structure-activity relationship studies, or *in-vitro* assays. In this thesis, AutoDock was used to calculate the binding site of quaternary ammonium ions in the pore of K2P channels. In order to predict the binding site, AutoDock [241, 242] searches possible ligand-protein conformations for configurations that maximise the the free energy of binding ΔG . The search space is includes any energetically possible conformation. The energetics of of each ligand-protein conformation are evaluated

using a semi-empirical force field, which uses parameters based on a large set of protein-inhibitor complexes for which both the complex structures and the inhibition constants are known. The calculation of ΔG consists of four independent calculations of:

- the change in intramolecular energy ($V_{bound}^{L-L} - V_{unbound}^{L-L}$) between the bound and unbound ligand conformation,
- the change in intramolecular energy ($V_{bound}^{P-P} - V_{unbound}^{P-P}$) between the bound and unbound protein conformation,
- the change in intermolecular energy ($V_{bound}^{L-P} - V_{unbound}^{L-P}$) between the bound and unbound ligand-protein complex conformation, and
- the loss in entropy upon ligand binding and the associated entropic cost ΔS_{conf} .

Here, each V is a pair-wise potential and includes terms for dispersion and repulsion, hydrogen bonding, electrostatics, and desolvation. P is the protein, and L is the ligand. The free energy of binding ΔG is the sum:

$$\begin{aligned} \Delta G = & (V_{bound}^{L-L} - V_{unbound}^{L-L}) \\ & + (V_{bound}^{P-P} - V_{unbound}^{P-P}) \\ & + (V_{bound}^{L-P} - V_{unbound}^{L-P} + T\Delta S_{conf}), \end{aligned} \tag{2.43}$$

and is calculated for each possible conformation. Each pair-wise potential V has the form:

$$\begin{aligned}
 V(i, j) = & W_{vdw} \sum_{i,j} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \right) \\
 & + W_{hbond} \sum_{i,j} E(\theta) \left(\frac{C'_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} \right) \\
 & + W_{elec} \sum_{i,j} \frac{q_i q_j}{\epsilon(r_{ij}) r_{ij}} + W_{sol} \sum_{i,j} (S_i V_i + S_j V_j) \exp \left(\frac{-r_{ij}^2}{2\sigma_{sol}^2} \right),
 \end{aligned} \tag{2.44}$$

where each term is multiplied by a weighting factor W , optimized by test sets fitted to experimentally determined inhibition data. The first term describes the van der Waals interactions between atoms i and j , with parameters A_{ij} and B_{ij} based on the Amber force field. The second term is a hydrogen bond potential with a maximal binding energy of 5 kcal mol⁻¹ at 1.9 Å for hydrogen bonds with oxygen or nitrogen and 1 kcal mol⁻¹ at 2.5 Å for hydrogen bonds with sulfur. Deviations from the optimum hydrogen bond geometry are incorporated by $E(\theta)$. The third term returns the interactions in a screened electrostatic of dielectricity $\epsilon(r_{ij})$. The fourth term is the desolvation potential, in which the volume of the surrounding atoms V_i times a solvation parameter S_j is weighted with a distance-dependent factor $\sigma = 3.5$ Å.

AutoDock allows rotatable ligand bonds, and a limited number of rotatable protein side chains. In this thesis, quaternary ammonium ions were docked into a large multiplicity of similar homology models, which allowed full pseudo-flexibility of the protein. Therefore, it was not necessary to use the AutoDock functionality of incorporating protein side chain flexibility.

Chapter 3

Computational studies of KirBac3.1

3.1 Introduction

Inwardly rectifying potassium (Kir) channels regulate the electrical membrane potential and are crucial for cellular function (Chapter 1) [5]. Kir channel activity modulates heart rate, controls insulin secretion, and regulates potassium homeostasis in the kidneys. Consequently, loss-of-function mutants in these channels cause inheritable cardiac arrhythmic disorders. A mutation in the Kir2.1 channel was found responsible for the Anderson-Tawil syndrome, which manifests itself in a reduced Kir2.1 current and in a prolonged period of depolarisation [243]. Besides, pathological deviations of Kir channel expression levels in non-excitabile cells or abnormal function of Kir channels can lead to neonatal diabetes, or to Bartter's disease, a rare, but severe, kidney condition [244, 245].

Therefore, Kir channels are important actors in physiological processes and understanding their mechanisms of gating is crucial to rationalize the development of targeted K^+ channel activators or inhibitors. Structural information of

Kir channels helps in this task. However, until 2009, next to several prokaryotic Ca^{2+} -dependent and voltage-gated K^{+} channels, crystal structures only existed of bacterial homologs of Kir channels, termed KirBac [246]. These structures identified a constriction at the helix bundle crossing (HBC) that has been proposed to form a mechanical gate (Fig 3.1a). Activation of this gate, termed the HBC gate, has been thought to involve an iris-like motion of the inner pore-lining transmembrane helices (TM2). Today, the HBC gate is believed to be allosterically coupled to conformational changes in the intracellular assembly (CTD). Mutations perturb gating by breaking the coupling between the TMs and the CTD and may lead to disease [247]. However, there are no simulations of X-ray structures of KirBac channels with the HBC gate in an open conformation.

In this chapter, molecular dynamics (MD) simulations are carried out using two new activatory mutant KirBac3.1 structures with an apparent open HBC gate. These simulations provide a dynamic mechanistic insight behind the activatory effect of these mutations, which derive from a random mutagenesis screen [248]. In these simulations, *in-silico* mutation of the mutated residues to the wild-type residue shows that these structures are indeed open at the HBC gate. MD simulations further address the mechanics and dynamics of Kir channel gating in at the HBC gate.

3.1.1 Gating in KirBac channels

The helix bundle crossing has been suggested as the primary activation gate of KirBac channels, and K^{+} channels are thought to share common mechanisms of pore opening [38, 250]. However, the linker between the transmembrane do-

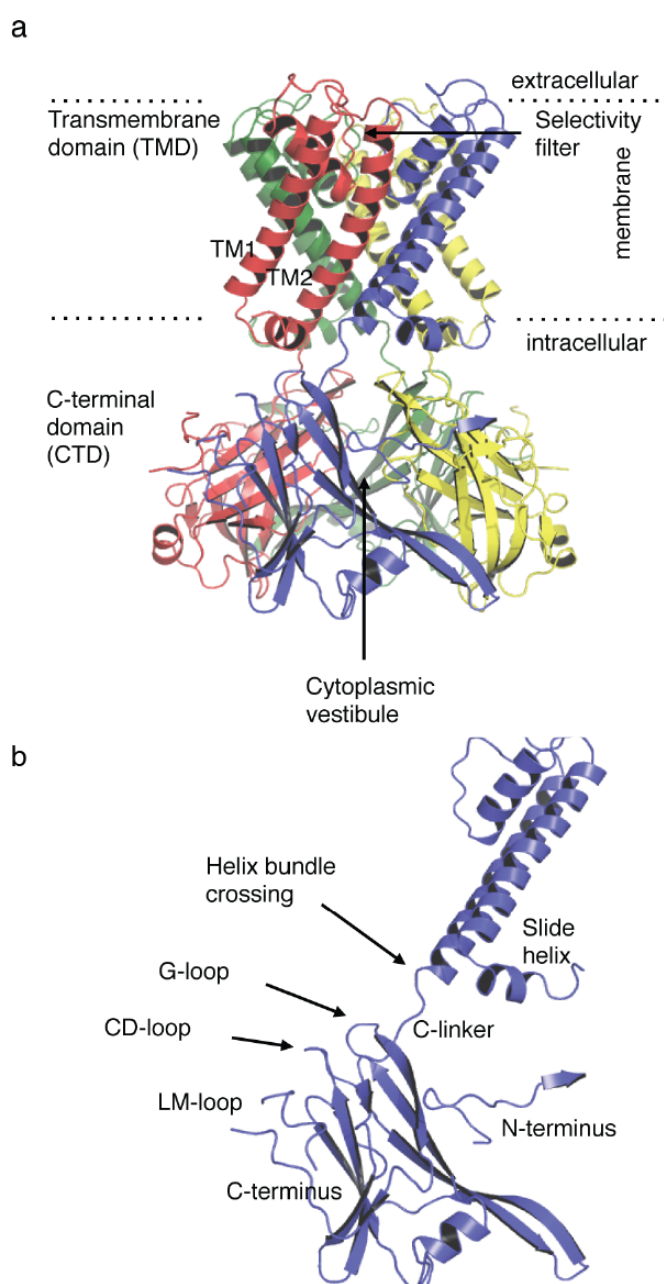


Figure 3.1: Overall structure of KirBac3.1, coloured by subunit. **(a)** Tetrameric assembly (PDB id: 4LP8, 2.46Å[249]). Transmembrane helices TM1, TM2, and the selectivity filter are in the transmembrane domain (TMD). The cytoplasmic vestibule is found in the C-terminal domain (CTD). **(b)** Single subunit of KirBac3.1. The helix bundle crossing (HBC), the CD-, and G-loop are indicated. The C-linker connects the TMD with the C-terminal domain. N-terminus and LM-loop are not fully resolved. The slide helix is at the membrane interface.

main (TMD) and the cytoplasmic assembly (CTD), as well as two loops on the the interfacial side of the CTD, the G-loop and CD-loop, were found in both KirBac and Kir channels to be sensitive to modulatory ligands such as cholesterol or PIP₂ (Fig 3.1b). Therefore, if the HBC is the primary gate, these cytoplasmic regulatory sites also have to couple to the TMD in order to enable opening of the HBC. Recent structures of eukaryotic Kir channels support this allosteric coupling hypothesis between the CTD and the TMD. However, although large conformational changes were observed in CTDs of these eukaryotic Kir channel structures, the HBC gate is still trapped in a closed, or 'pre-open' conformation [65, 58, 251]. Furthermore, a series of KirBac structures suggests that twisting of the CTD leads to gating of KirBac at the selectivity filter, bypassing the HBC. Moreover, in all simulations, and X-ray crystal structures of Kir or KirBac channels so far, the HBC is closed [64].

Thus, the exact mechanism of Kir channel gating remains hypothetical, and the extent of coupling between CTD and TMD, or the extent of opening at the HBC remains to be established. The Tucker group solved the X-ray crystal structure of a KirBac3.1 channel in an apparent open conformation (PDB id: 3ZRS [252]). Here, this structure is shown to be indeed open and electrostatically attractive for K⁺ at the HBC (Chapter 3.3). By comparison of the open KirBac3.1 conformation with the closed structures, mechanisms of coupling between the CTD and the TMD are explored. Thereafter, this structure is compared with an X-ray structure of a KirBac3.1 channel in an alternative open conformation (Chapter 3.4). MD simulations show that this open structure must represent a high-energy state. Furthermore, MD simulations of these two new open structures, show that the G-loop of KirBac channels acts as mechanical gate, which

is notable since it is open in all existing bacterial Kir structures. In KirBac3.1, this G-loop gate appears to be only weakly coupled to the HBC gate. The MD simulations display transition steps between open and closed KirBac channels conformations, complementing the high-resolution structural information.

3.1.2 In-vivo and in-silico activatory mutants

As conventional methods to crystallise KirBac in an open conformation have failed, the Tucker group scanned KirBac3.1 for activatory mutations by expressing randomly mutated KirBac3.1 in auxotrophic (K^+ -uptake deficient) *E. coli* (Figure 3.2). Those mutants which complemented growth in low- K^+ media are likely to have an activatory effect and were used for subsequent crystallisation trials. Notably, one cluster of mutants localised at the HBC gate (Figure 3.2a, b), and the HBC gate opened in KirBac6.1-G137D MD simulations [248] as well as in KirBac1.1-G143E simulations [253]. Therefore, the corresponding activatory KirBac3.1-S129R mutation (Figure 3.2c) was crystallised, in the expectation to obtain a structure which is open at the HBC. The KirBac3.1-S129D mutant appears to be even more active than the S129R mutant.

Several activatory mutants obtained in the random mutagenesis screen are found in the CTD (Figure 3.2a, d); Clarke and Gulbis hoped that their structures would provide insights into the coupling mechanisms of CTD and the TMD. However, their KirBac3.1-Q170A CTD-mutant structure crystallised in a closed conformation. Therefore, the Tucker group solved the structure of the KirBac3.1-S129R/S205L double mutant (Figure 3.2d), knowing that both S129R (Figure 3.2c) and S205L (Figure 3.2d) mutant channels complement growth, to examine the impact of the activatory cytoplasmic S205L mutation in the context of an

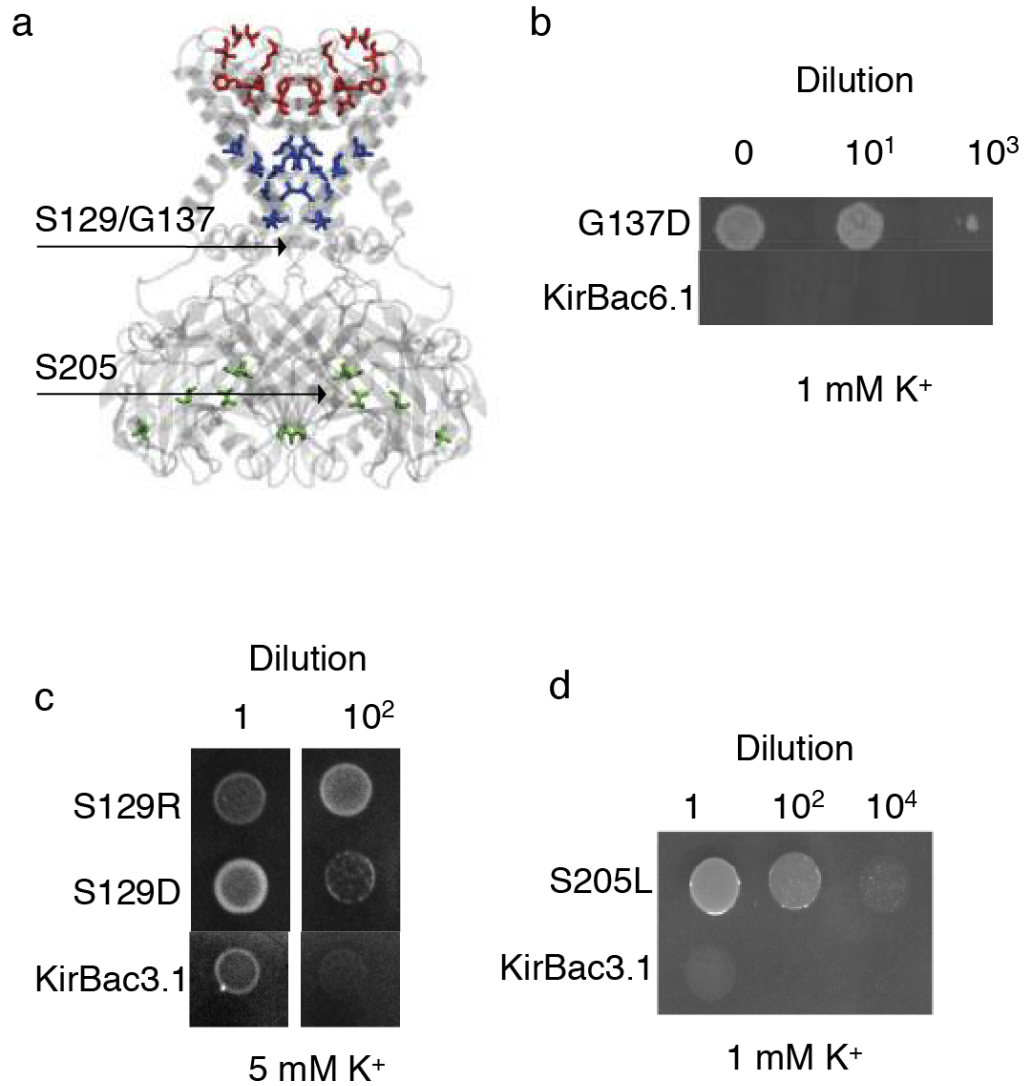


Figure 3.2: Activatory mutants in KirBac. (a) Two clusters are observed, one in the transmembrane domain (TMD) and in the cytoplasmic domain (CTD). (b-d) Auxotrophic, (K⁺-uptake deficient) KirBac-mutant channels complement growth in 1mM K⁺ or 5mM K⁺ for (b) G137D (KirBac6.1), (c) S129R/S129D at the helix bundle crossing, and (e) at the intracellular cytoplasmic subunit interface. Adapted with permission from [252, 248].

open HBC gate.

The effect of the S205L mutation on the CTD is examined in Section 3.4 using MD simulations of the KirBac3.1-S129R and the KirBac3.1-S129R/S205L structures. Previous simulation studies of the KirBac3.1-S129D and the KirBac6.1-G137E (Figure 3.2b) mutants, although pointing out opening at the HBC gate, did not address the coupling of conformational changes between the CTD and the HBC, nor was the electrostatic pore profile in the context of an open KirBac channel examined [248, 253]. Poisson Boltzmann calculations have found, however, that H219 in the (closed) KirBac1.1 structure contributes significantly to the electrostatic field in the CTD pore [254]. Thus it appears reasonable that the corresponding activatory mutant KirBac3.1 S205L represents a more conductive state. Interestingly, several other residues that contribute $> 1 \text{ kcal mol}^{-1}$ to the electrostatic field in KirBac1.1 or in homology models of Kir [254], also complemented growth[248].

3.2 Methods

In this section, the specific parameters used in the simulations, and the details of the electrostatic calculations and modelling procedures are summarised.

3.2.1 Modelling

All models used for molecular dynamics simulations are based on two crystal structures: the KirBac3.1 structure locked into an open state via the S129R mutation at the HBC (PDB id: 3ZRS, 3.05 Å resolution) and the KirBac3.1 S129R-S205L double-mutant structure (PDB id: 4LP8, 2.46 Å resolution). Modeller9.9

[225] was used to add missing residues to the N-terminus of the 3ZRS structure and the resulting model was used as an input for a 100 ns molecular dynamics simulation (model “R129”).

To determine the influence of the mutations S129R and S205L on the dynamics of KirBac3.1, two models with wild-type sequence were generated, based on either the coordinates of the 3ZRS (model “R129S”) or the 4LP8 (model “R129S-L205S”) structure. Since the side chain of serine is similar or smaller in size than a leucine or arginine side chain, the mutant residues could be modelled as serines without steric clashes. In summary, the following models were used for atomistic (AT) MD simulations: R129 (1 x 100 ns), R129S (2 x 100 ns), R129S-L205S (2 x 100 ns).

3.2.2 Poisson-Boltzmann calculations

The electrostatic potential and its contribution to the energy profile of K^+ ions passing through the cytoplasmic pore of the crystal structures was calculated using the APBS package [235, 255]. Charges and radii were assigned using PDB2PQR [256], which was also used to add missing atoms to all unresolved side chains before calculating electrostatic energies. Energies were calculated using a NaCl bath with an ionic strength of 0.2 M at 298 K. The protein (dielectric constant $\epsilon = 10$) was embedded into a dielectric slab ($\epsilon = 2$), mimicking the membrane environment, with the membrane thickness set to 45 Å. The pore itself was excluded from the membrane (exclusion radius $r = 5$ Å) and assigned a solvent dielectric ($\epsilon = 80$). The C_α atom of R129 in the 3ZRS structure was set to $z = 0$ Å and the structure aligned along the selectivity filter of 3ZRS. The cytoplasmic side of the dielectric membrane slab is delimited at $z = -5$ Å, close

to the tip of the R129 guanidino group ($z = -7 \text{ \AA}$). A step size of $\Delta z = 1 \text{ \AA}$ was used for the calculations.

3.2.3 Molecular dynamics simulations

All simulations were carried out using GROMACS4.5.4 [184]. Each model was embedded into a neutral palmitoyl-oleoyl phosphatidyl choline (POPC) phospholipid bilayer during a coarse grained (CG) self-assembly simulation (100 ns) using the MARTINI2.1 force field [197, 213]. In the MARTINI force field, POPC lipids are modelled through a positive CG particle (choline) and a negative CG particle (phosphate), which are linked by two polar particles (glycerol) to two acyl chains comprising five or four hydrophobic CG particles, respectively. To neutralize any electrical net charge of the protein, monovalent Cl^- and Na^+ ions were added to the solvent. A Gaussian network model with a $10 \text{ \AA}$ cut-off and an elastic bond force constant of $1,000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ retained the tertiary and quaternary structure of the channel during CG simulations [215].

Final CG snapshots were converted into atomistic systems using a fragment-based approach and two K^+ atoms were added to the selectivity filter at positions S2 and S4 to simulate a stable, conductive state [257]. The cavity below the selectivity filter was solvated using a grid-based approach using VOIDOO and FLOOD [258], in order to prevent collapse of the pore. Each atomistic system was energy-minimized using at least 5,000 iterations of the steepest descent or limited-memory Broyden-Fletcher-Goldfarb-Shanno (l-BFGS) quasi-Newtonian minimizer to relax steric clashes [259, 260]. The system was minimized during a 1 ns atomistic simulation with positional restraints of $1,000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ applied to all heavy atoms of the protein. This ensured equi-

libration of the solvent and, to an extent, of lipid-protein contacts. For each equilibrated system, an atomistic simulation of 100 ns duration was performed.

Each CG simulation comprised ~ 270 lipids and $\sim 40,000$ water molecules. A time-step of $\Delta t = 20$ fs was used, and atomic coordinates written out every 100 ps. A cut-off of 12 Å was used for Lennard-Jones and electrostatic interactions. The neighbour-search cut-off was set to 13 Å, to accommodate for the size of charge groups and diffusion between neighbour updates. The system was coupled to a constant heat bath with $T = 323$ K using the Berendsen thermostat [206], with a coupling constant of $\tau_T = 1$ ps. The pressure was set to $p = 1$ bar by semi-isotropic exponential relaxation of the pressure coupling, with a coupling constant of $\tau_P = 1$ ps and a compressibility of $\kappa = 5 \times 10^{-6} \text{ bar}^{-1}$.

Atomistic simulations were carried out using the GROMOS96 43a1 force field, Berger lipid parameters, and the SPC water model [186, 196, 198]. With a time step of $\Delta t = 2$ fs, coordinates were written out every 10 ps for analysis. Parameters for atomistic (AT) simulations were the same as for CG simulations, except that a 10 Å short-ranged cut-off was used for Lennard-Jones interactions and electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method with a real-space Coulomb cut-off of 10 Å [203, 205, 204]. In the AT simulations, the temperature was regulated via a velocity rescaling method, with $\tau_P = 0.1$ ps and Parrinello-Rahman pressure coupling was used with $\kappa = 4.5 \times 10^{-5}$ [209]. Bond lengths were constrained using the LINCS algorithm [200].

3.3 Structure of KirBac-S129R

The KirBac-S129R X-ray structure contained 280 out of 301 residues. Although being the most complete KirBac structure, residues in the β LM-loop and in the N-terminus could not be resolved. Hence, for the simulations and the analysis of this structure, the missing residues were modelled *de novo* using Modeller9.9. In total, 20 models were generated and the model with the best Modeller score was selected for MD simulations. The pore radius, as described by HOLE profiles [261], *in-silico* mutations, and comparison of the KirBac3.1-R129S structure with *in-silico* mutants highlight major conformational changes in the TMD, and in particular at the HBC (Figures 3.3a, b).

3.3.1 The S129R structure is open at the HBC

HOLE profiles demonstrate that the pore of the helix bundle crossing (HBC) in the S129R mutant structure is trapped in an open state, while it is fully closed in all other KirBac channels (Figure 3.3) [262]. In all KirBac structures, side chains of aromatic residues such as tyrosine or phenylalanine sterically impede the ion conduction pathway at the HBC. In KirBac3.1-S129R however, the Y132 C $_{\alpha}$ -C $_{\alpha}$ distance opens up to 17.2 Å, while it ranges from 12.2 Å to 13.5 Å in closed-state KirBac structures (Figure 3.3b). The R129 side chain at the HBC obstructs the ion pathway (Figure 3.4), but since R129 is a serine in wild-type KirBac3.1, and a glycine in all other KirBac channels, the mutant structure was analysed with a serine in place of R129. With this *in-silico* replacement, the pore is then found to be open (pore radius >5 Å from HOLE [261]) at the HBC (Figure 3.3c, d), in contrast to all other KirBac structures available to date.

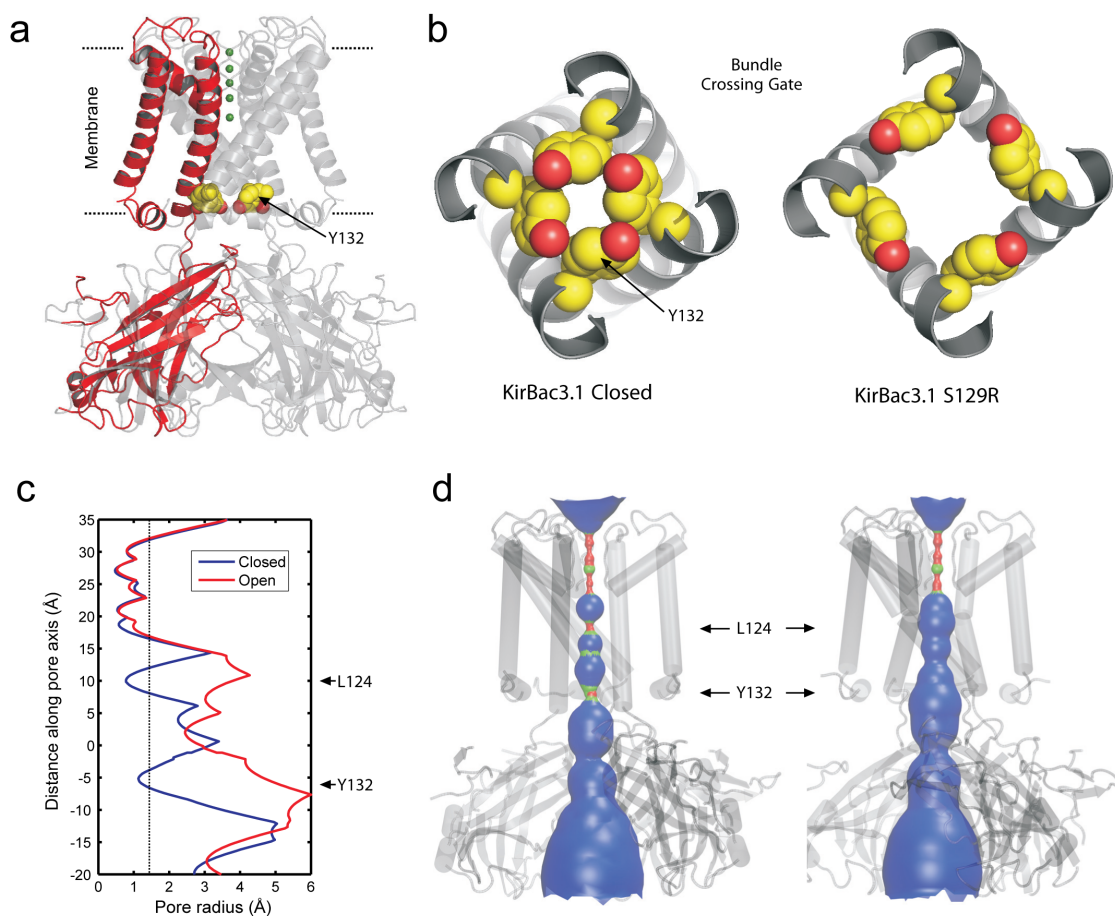


Figure 3.3: KirBac3.1 trapped in an apparent open state. **(a)** KirBac3.1-S129R crystal structure. **(b)** closed and open helix bundle crossing (Y132) for closed (2WLJ) and open conformation. **(c)** Pore radius of the open state, in which the side chain of R129 has been changed back to a serine, along the vertical axis, computed by HOLE [261]. Constrictions at the secondary gate, L124, and Y132, the helix bundle crossing, are pointed out. **(d)** Closed state (2WLJ, [64]) compared to open state structure.

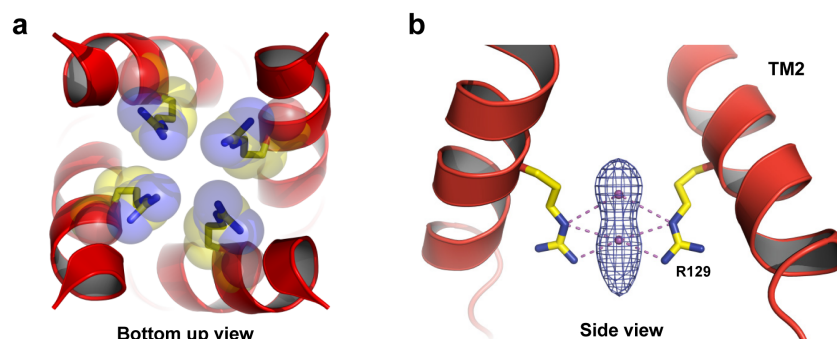


Figure 3.4: Location of the S129R mutation. (a) Bottom-up view from the helix bundle crossing as seen from the cytosolic pore. The R129 mutant side chain, which is a glycine in most other Kir or KirBac channels, points towards the pore, blocking the ion conduction pathway. (b) Cl^- ions are coordinated by R129. The electron density (by Bavro and Tucker, $F_O - F_C$ OMIT map contoured at σ 2.7) is in good agreement with MD simulations and APBS calculations. Images made with Pymol [35].

This opening is independent of the wild-type serine rotamer chosen for the *in-silico* replacement of R129. Therefore, the R129 mutant appears to stabilize the transmembrane helices in an open conformation.

3.3.2 Bending at hinge glycine in the TM helices opens the HBC

The changes that unfold in the TMD as a result of the opening at the HBC are in agreement with previous modelling studies of KirBac3.1 [263, 264, 62], opening of the HBC gate in crystal structures of KcsA [265] and more recent simulation studies of KcsA [253] (Figure 3.5). While the upper part of the TM2 remains relatively stable, the splaying of the TM2 at the HBC, is concomitant with a 20° hinging or bending at the conserved hinge-glycine G121 (Figure 3.5a). In addition, the lower part of the TM2 twists along its helical axis by circa 25° (Figure 3.5b). The position of the Y132- C_α atoms fits well with the corresponding residue T122 in the 17 Å open KcsA crystal structure (Figure 3.6)

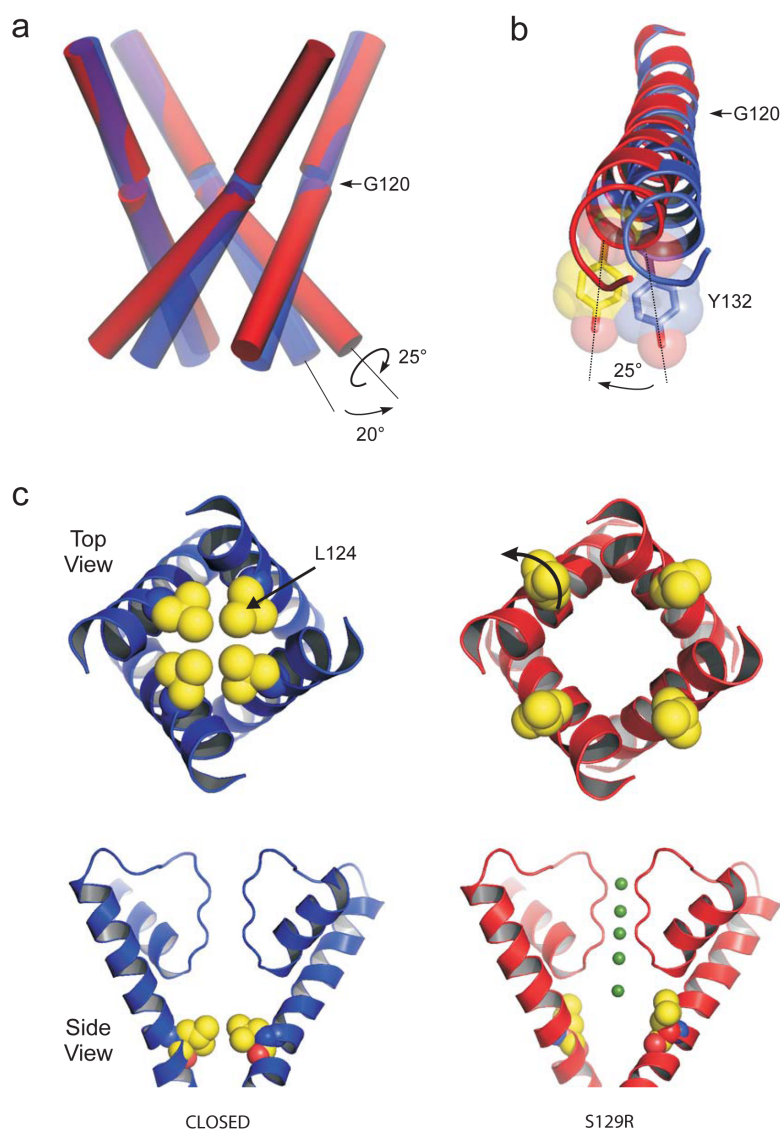


Figure 3.5: Bending and twisting at the inner helices during channel opening. **(a)** Comparison of the inner pore lining helices (TM2) of the open KirBac3.1 structure (red) and a closed structure (blue). The TM2s bend at the conserved hinge glycine G120 by 20° and rotate clockwise along their helical axis by approx. 25° during channel opening. **(b)** Rotation of the lower end of TM2 during opening. Side chains of the open and closed state are shown as spheres and sticks.

[266, 267].

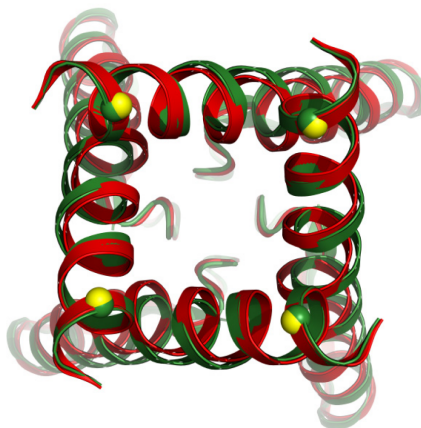


Figure 3.6: Overlay of the open helix bundle crossing of KirBac3.1 (shown in red) with the 17 Å open KcsA structure (shown in green). Alignment of the inner transmembrane helices (TM2s) of the open KirBac3.1 with KcsA (PDB id 3F7Y, [266]) gives good fit (RMSD < 0.9 Å). The 17.1 Å opening at T112 in KcsA (C_{α} atom shown as green spheres) is in good agreement with the 17.2 Å opening at the corresponding A133 residue (C_{α} atoms shown as yellow spheres) of TM2 in KirBac3.1.

3.3.3 Rotation of inner TM helices opens a secondary electrostatic gate

Together with bending of the TM2 helices at the G120 hinge residue, the TM2 helices rotate by 25° around their vertical axis. This movement turns the side chain of L124, which forms part of a secondary gate in KirBac3.1 [268, 64] away from the pore, unblocking the ion conduction pathway (Figure 3.5c). As a result, the radius of the pore below the selectivity filter increases from less than 0.6 Å to >4 Å (Figure 3.3c). This is important, as the pore along the entire ion pathway is now fully permeable to K^{+} ions (Figure 3.3d).

It is noteworthy that the corresponding residue of L124 in the eukaryotic Kir2.1 channel is the rectification control site D172. Charge neutralization of this site below the selectivity filter weakens inward rectification and diminishes

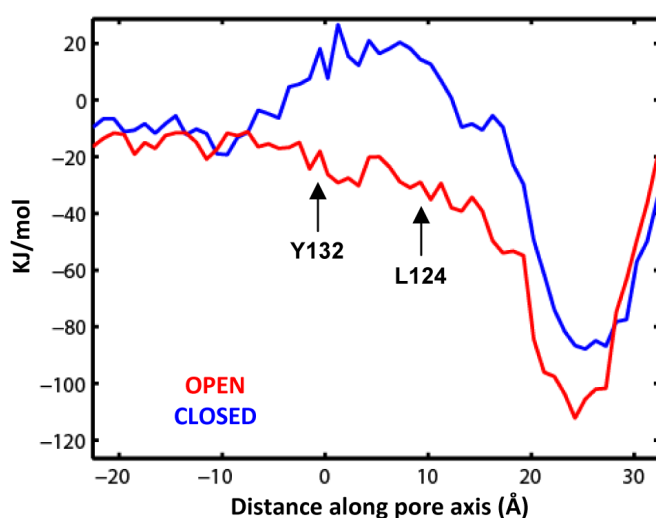


Figure 3.7: APBS calculations of the electrostatic energy profile along the ion conduction pathway. Compared are the electrostatic energy profiles of K^+ permeation for a wild-type closed state KirBac1.3 (PDB id 2X6C [64]) structure and the open conformation of the S129R mutant in which the arginine side chain has been replaced by a serine, which is found in the wild-type channel. The energetic barrier for permeation is removed in the open conformation and the path for permeation becomes gradually more favourable for K^+ when moving from the intracellular side towards the selectivity filter.

Mg²⁺ binding affinity in these channels [269]. Interestingly, Poisson-Boltzmann calculations reveal a strong decrease in the energetic barrier of K⁺ ions at this position (Figure 3.7). This contributes to help K⁺ ions to permeate past the HBC gate, towards the selectivity filter. A decrease in the energetic barrier was also seen for all other KirBac structures (not shown). However, a correlation of the energetic barrier with the twisting status was not observed. Nevertheless, these data show that the open KirBac3.1 structure, in which the mutant arginine side chain at the HBC is replaced by the wild-type serine, is electrostatically conductive.

3.3.4 Conductive selectivity filter despite twisted CTD

In a series of closed KirBac3.1 structures, the CTD conformation is either twisted or untwisted relative to the vertical pore axis (Figure 3.8). The gating model put forward by Clarke *et al.* proposes that twisting of the CTD with respect to the TMD is directly correlated to the conductivity status of the selectivity filter [64]. According to this model, twisting of the CTD is correlated with a non-conductive selectivity filter. In direct contradiction to this model the K⁺ ions can be modelled into the electron density at all four ion binding sites S1 - S4 [257] although the CTD in KirBac3.1-S129R is twisted (Figure 3.8). This indicates that the status of the selectivity filter in the twisted S129R structure is conductive [257].

3.3.5 Network links twisting of CTD to opening at the HBC

The new KirBac1.3-S129R conformation directly couples movement of the CTD to opening at the HBC gate. This coupling is demonstrated by a network of

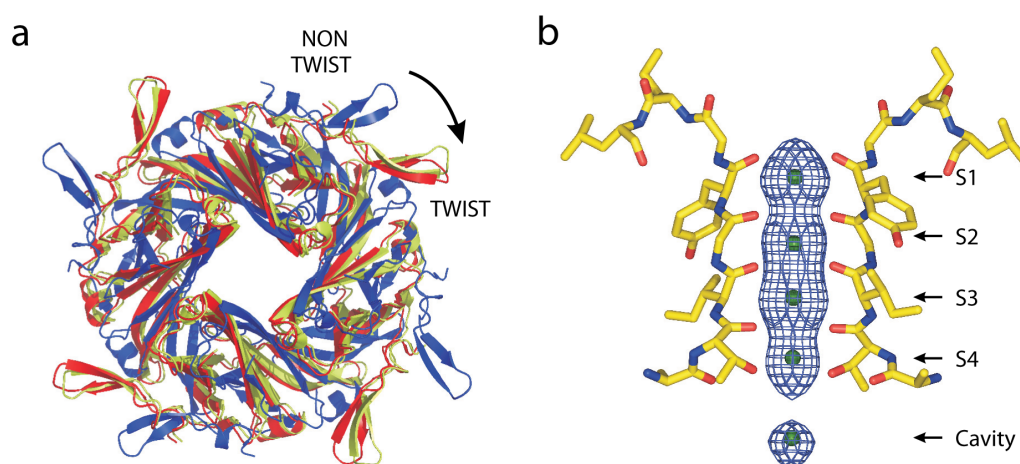


Figure 3.8: Twisted cytoplasmic assembly, yet conductive selectivity filter. (a) The open S129R conformation (red) compared to a wild-type KirBac1.3 closed structure (2WLJ, blue) and a twist (2X6C, yellow) structure, as seen from the transmembrane domain (TMD, not shown), aligned along the TMD. The open S129R conformation is twisted. (b) Ion coordination sites resolved (by Bavro and Tucker) in the selectivity filter of the open S129R structure are indicative of a conductive selectivity filter.

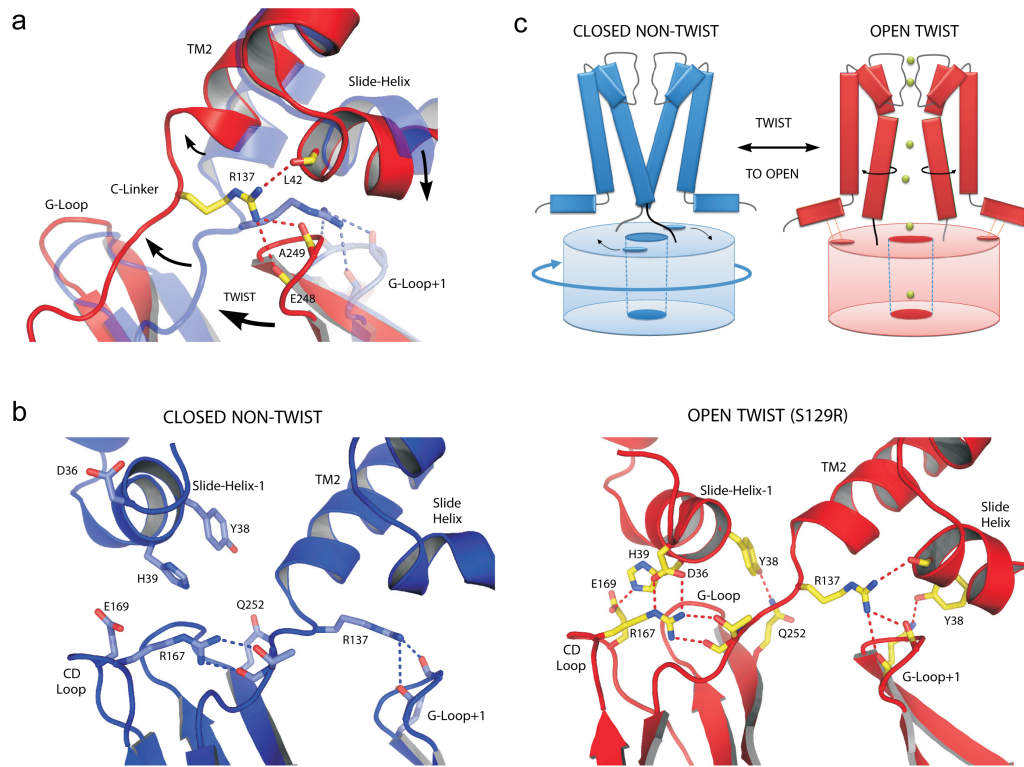


Figure 3.9: Structural network coupling the CTD to the TMD. **(a)** A more extensive network of interactions is observed in the open S129R mutant conformation (red) compared to the wild-type closed KirBac1.3 structure (PDB id 2WLJ). The C-linker interacts with the backbone carbonyls of the G-loop of the adjacent subunit (G-loop+1). In the open S129R twist conformation, the C-linker is displaced and forms an additional intrasubunit bond with the slide helix. **(b)** Comparison between the interactions between the CTD and TMD in the closed wild-type KirBac3.1 and the open S129R mutant conformation. Movement of slide helix and C-linker are enabling an interdependent network of coupled domains. **(c)** Proposed model of KirBac gating. Rotation of the CTD brings the C-linker and the CD loops (shown as small spheres on top of the CTD) into register with the slide helices, thereby coupling rotational movement of the CTD to the opening at the HBC.

inter- and intrasubunit interactions in the open state S129R structure (Figure 3.9). Notably, rotation of the CTD displaces the C-linker, which could be conducive to opening at the HBC (Figure 3.9a). The side chain of R137 firmly anchors the C-linker to the backbone of the G-loop of the adjacent subunit. Although this interaction is also observed in the closed structures, the S129R open structure exhibits an additional subunit interaction between the side chain of R137 and the backbone of the slide helix (Figure 3.9b). The backbone of the C-linker forms an interaction with the R167 side chain on the CD loop of the same subunit, which is also observed in the closed state structures. In the KirBac-S129R structure however, a twist in the slide helix allows formation of a salt bridge between R167 on the CD loop and the conserved D36 on the slide helix. This connection is supplemented by another polar interaction between Q169 on the CD loop and H39 on the slide helix. These additional interactions could help to promote the stabilisation of a twisted CTD and an open HBC (Figure 3.9c). Notably, in mammalian Kir channels, the role of the CD loop is crucial for ligand regulation [270, 271]. This includes cholesterol, which binds directly to prokaryotic Kir channels and has which has complex effects on a large number of ion channels [271]. Next, MD simulations will give further information on this network.

3.4 The double-mutant KirBac3.1-S129R-S205L

The structure of the KirBac-S129R activatory mutant highlighted structural changes in the CTD and the TM2 that are primarily associated with a pore radius increase of >4 Å at the HBC. MD simulations of the activatory mu-

tant KirBac1.3-S129R-S205L structure examine a second pathway to activation. These simulations show that introduction of the S205L mutant into KirBac1.3-S129R leads to reversible changes in inter-subunit interfaces that have a significant effect on the electrostatic conductivity of the CTD pore.

3.4.1 X-ray crystal structure of KirBac3.1-S129R/S205L

The S129R/S205L activatory KirBac3.1 mutant was solved at 2.46 Å resolution (by Zubcevic and Tucker). When aligned to the S129R (PDB id 3ZRS) structure, we found that this double-mutant structure exhibits an RMSD <0.3 Å between matching residues. Despite this high similarity, conformational changes in the inter-subunit interfaces in the CTD are apparent (Figure 3.10). The triad of inter-subunit side chain interactions between E173, D175 and H177 is found in all KirBac1.3 structures to date (Figure 3.10a). However, in the S129R/S205L structure, a change in the rotameric conformation of the H177 side chain disrupts this triad and leads to formation of a set of inter-subunit connections between H177 and D197 on one subunit, and R202 on the adjacent subunit (Figure 3.10b, c). The net effect of the rotameric side chain change of H177 is a concerted realignment of the charged side chains of E173 and D175 forming a ring of charges protruding towards the inside of the cytoplasmic pore (Figure 3.11a). This mechanism contributes to a lower electrostatic potential [272], which leads to stronger rectification in eukaryotic Kir channels. Consequently, it was possible to model a K⁺ ion into the electron density at the centre of this ring, which is consistent with the cation binding site found in the Kir2.2 and Kir3.1-S225E structures (Figure 3.11b, c) [65].

Interestingly, the inter-subunit interactions in the R129S/S205L structure

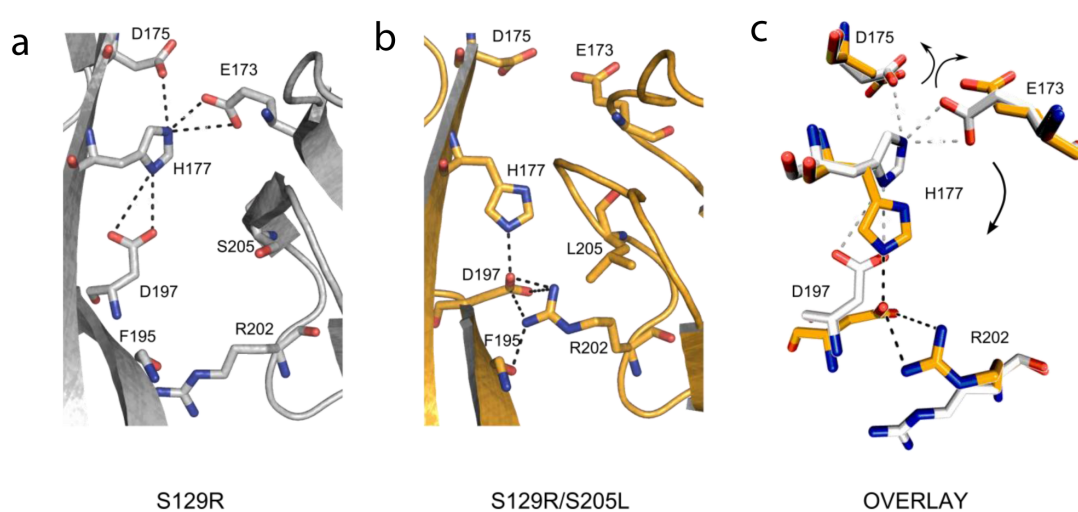


Figure 3.10: Rearrangement of intersubunit contacts in the S205L mutant. (a) Subunit interface of the R129S mutant, in which E173, D175 and H177 form a triad of interactions confined towards the upper end of the subunit interface. (b) In the S129R/S205L structure, a change in the rotameric conformation of the H177 side chain breaks the E173-D175-H177 triad and leads to formation of a more extensive intersubunit network, spanning from the strengthened R202-D197 salt bridge in to the charged side chains of E173 and D175 which are now pointing towards the cytoplasmic pore axis. (c) Overlay of S129R and S129R/S205L mutant structures.

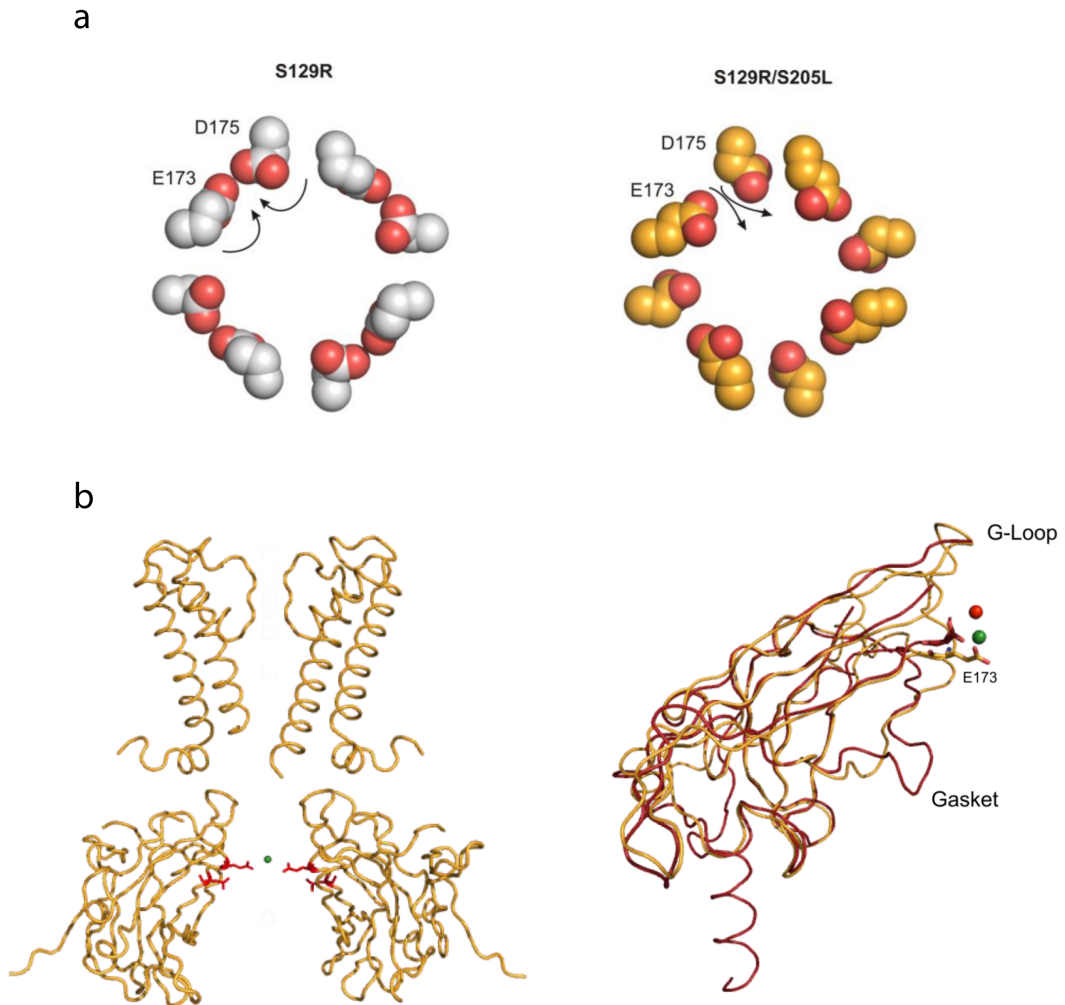


Figure 3.11: S205L mutation results in a ring of negatively charged side chains pointing towards the cytoplasmic pore. **(a)** Top-down view of the arrangement of E173 and D175 residues relative to the cytoplasmic pore in the S129R (grey, left) and in the S129R/S205L (gold, right) structures. In the S129R structure, E173 and D175 are confined to the intersubunit space. Introduction of the S205L mutation releases them from interacting with H177 and enables them to point towards the inner vestibule. **(b)** Alignment of the cytoplasmic domains of KirBac3.1 S129R/S205L (gold) and Kir3.1 S225E mutant (red) structures. The S225E mutation mimics the E224/5 residue which is found in the strongly inward-rectifying Kir2.1 and Kir2.2 channels. In Kir channels, this residue forms a cation binding site similar to E173 and D175 in KirBac. The Na^+ ion found in the Kir3.1 S225E structure is shown as a red sphere, while the K^+ ion found in the S129R/S205L mutant is shown as a green sphere. By facilitating polyamine block, this binding site is important for inward-rectification in Kir channels.

are supplemented by an additional backbone-side chain interaction between F195 and R202. These residues delineate the binding pocket for co-crystallised polyamines in latched interfaces of closed-state structures [64]. Staged changes in the CTD in a series of closed state structures were interpreted as releasing polyamines from the binding pocket into the pore [64, 273]. However, whether the R129S/S205L CTD interfaces are latched or unlatched has to be subject of further studies.

3.4.2 Mechanism of activation by S205L

The electrostatic potential inside the cytoplasmic vestibule determines the cationic ion concentration. To test whether the electrostatic potential is affected by the S205L mutation, Adaptive Poisson Boltzmann Solver calculations were carried out, through the CTD, along the axis of the ionic conduction pathway of both the S129R/S205L and the S129R mutant structures. Compared to the S129R structure, APBS profiles of the S129R/S205L mutant show a significant decrease in electrostatic potential at the level of E173 and D175 (Figure 3.12). Thus, the S205L mutant appears to lower the energetic barrier for K^+ ion permeation in the pore.

Interestingly, Poisson-Boltzmann calculations by Robertson *et al.* show that in a Kir2.1 and Kir3.1 model, the residues corresponding to S205 and R257 contribute strongly to the electrostatic potential [254]. In KirBac3.1, R257 on the β H sheet forms an inter-subunit salt bridge with E248 on the G-loop, and the E248 backbone links this network of interactions with the side chain of R137 on the adjacent C-linker. R257 (and E248) may therefore play a crucial role in KirBac3.1 gating, and indeed, the corresponding residue in KirBac6.1,

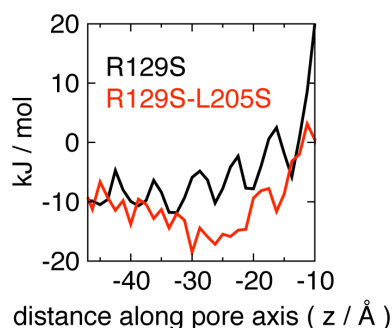


Figure 3.12: APBS profiles of the S129R and S129R/S205L structures demonstrate electrostatic changes in the ion conduction pathway of the cytoplasmic pore. The C_{α} -R129 positions correspond to $z = 0$. The Poisson-Boltzmann potential energy of a K^{+} ion, calculated along the pore axis of the CTD, is lower in the S129R/S205L than in the novel S129R structure. The decrease in energetic potential allows easier permeation of K^{+} ions through the CTD pore in the S129R/S205L mutant structure compared to the S129R and wild-type structures.

R265, activates KirBac6.1 when neutralized [248]. The same arguments may be applicable to other residues in the CTD.

3.4.3 Stability of the CTD in MD simulations

To examine whether the structural changes introduced by the S205L mutation have any other effects on the CTD, a series of atomistic molecular dynamics simulations was performed. During a 100 ns simulation of the S129R mutant (PDB id 3ZRS), the structure appeared to remain stable and no further opening (or closing) was observed, indicating a high degree of stability (3.9 Å C_{α} -RMSD after 100 ns). Therefore, it was decided to run 100 ns long simulations of the S129R and S129R/S205L open-state structures with the mutant side-chains changed back to the wild-type side chain, i.e. KirBac1.3 R129S and KirBac1.3 R129S/L205S. This would allow any constraints keeping these mutant struc-

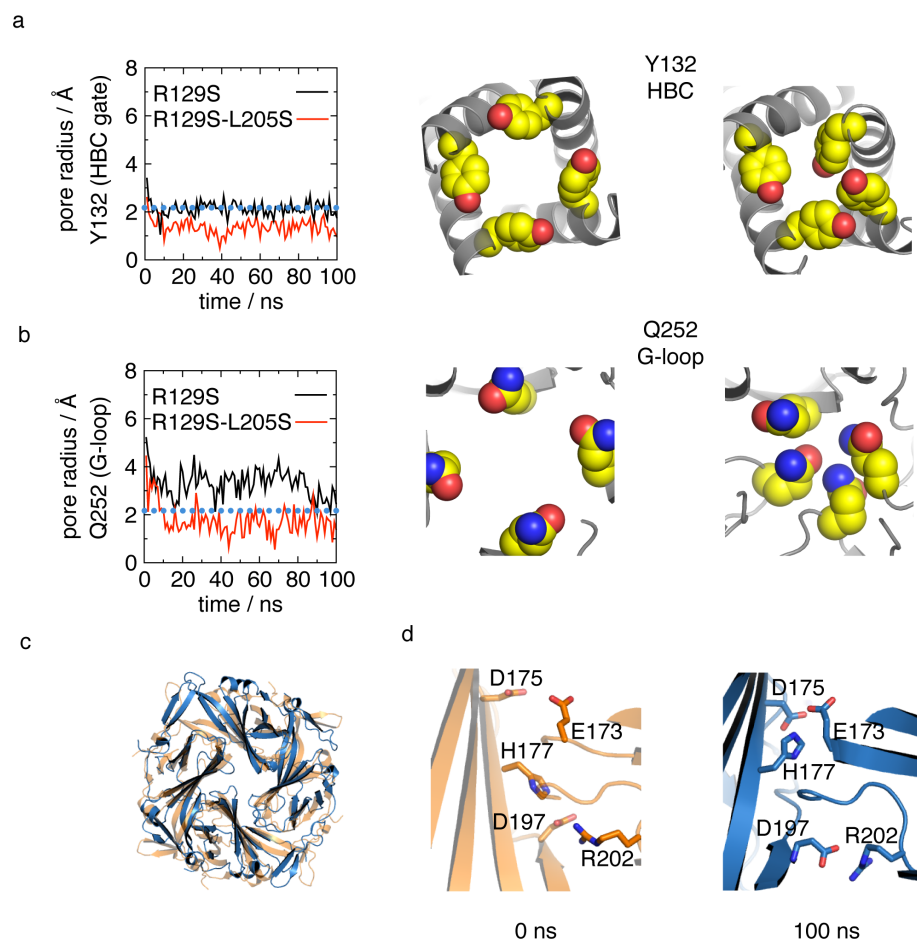


Figure 3.13: S205L and the effect on the stability on the open state conformation during molecular dynamics (MD) simulations of the single-mutant ("R129S") and the double-mutant ("R129S-L205S") structures. The mutant residues were converted back to wild-type serines. The pore radius, calculated by HOLE [261] along the pore axis, is shown on the left, for (a) the HBC (Y132), and (b) the G-loop (Q252). The radius of a hydrated water K^+ ion is indicated. For simulation times 0 ns and 100 ns, snapshots of the respective locations obtained in the R129S-L205S simulation are shown on the right. While the G-loop closes in the R129S-L205S simulation only, the HBC closes in all simulations. (c) Overlay of the CTD of the open double-mutant structure and of a snapshot after 100 ns MD simulation. No rotation is observed in the CTD. (d) Side-by-side view of the subunit interface in the CTD of the R129S-L205S simulation. Initially, side chains of E173 and D175 are pointing towards the pore and the H177 side chain is pointing downwards, interacting with D197 and R202. After 100 ns, H177 adopts the closed-state conformation (in three out of four interfaces). The side chains of E173 and D175 follow and turn away from the pore. This describes a "reverse" transition from an open to a closed state CTD conformation.

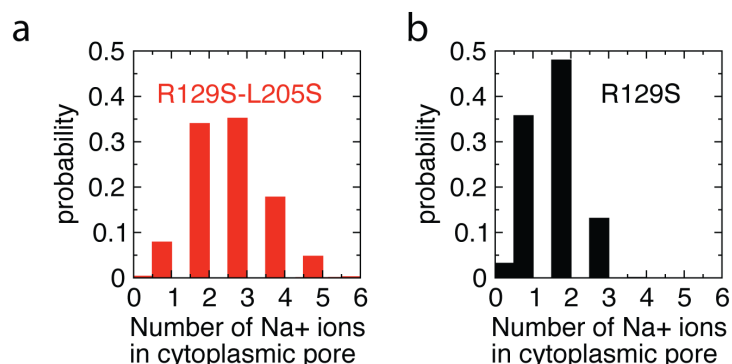


Figure 3.14: Number of cations (Na⁺) in the cytoplasmic pore ($-16 \text{ \AA} < z < -37 \text{ \AA}$, where the C _{α} -R129 positions correspond to $z = 0$) in the R129S/L205S simulation and the (b) R129S simulation. The cytoplasmic pore of the double mutant R129S/L205S allows a higher number of cations than the R129S mutant.

tures open to be released, and enable observation of any potential relaxation back to the closed conformation.

Intriguingly, during these simulations, major conformational changes were observed. In all of the simulations, the HBC gate started to close due to reorientation of the TM2 and the Y132 side chain (Figure 3.13a). Similar closure was also seen at the upper constriction in TM2. However, a major difference was noted at the G-loop located just below the HBC gate, which has been proposed to act as an additional gate within the CTD [274, 244, 275]. Closure of the G-loop gate was evident during the simulation of the double mutant structure (R129S/L205S), but not of the single mutant structure (R129S) (Figure 3.13b). Closure of the G-loop reduced the radius of the conduction pathway from 5 Å to $< 2.2 \text{ \AA}$ i.e. well below that of a hydrated K⁺ ion. These results suggest that the putative G-loop gate closes much faster in the double-mutant structure and that this new structure may therefore represent a higher-energy open conformation.

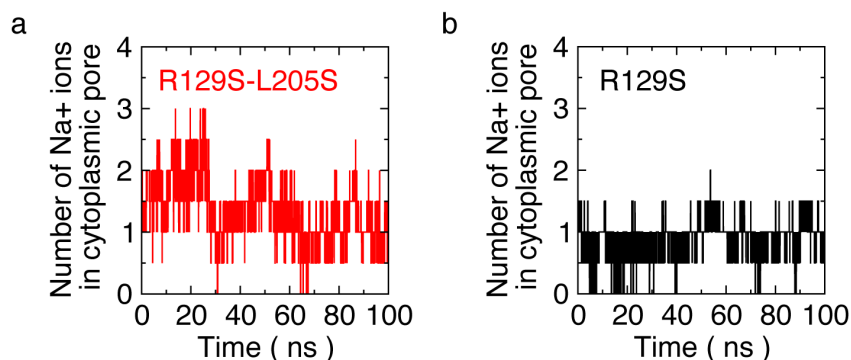


Figure 3.15: Ion occupancy in the CTD. (a) Number of cations (Na⁺) in the CTD during the S129R-S205L simulation. (b) Number of cations during the S129R simulation. Due to the reorientation of the charged side chains of E173 and D175 during the R129S-L205S simulation, the ion occupancy of the R129S-L205S simulation (red) diminishes towards the ion occupancy of the R129S simulation (black), which remains constant.

Do any changes occur at the CTD interface during the R129S/L205S simulation? No rotation of the CTD assembly relative to the TMD was observed in the double-mutant simulation, but the interface interactions did undergo a significant change (Figure 3.13c, d). The H177 side chain initially interacted with D197 and R202 (0 ns), but in three out of the four subunit interfaces, subtle twists occurred in the G β and H β strands, which disrupted the interactions between H177 and D197/R202, and enabled H177 to interact with E173 and D175. This reverted these negatively charged side-chains to their conformation observed in the original S129R structure (PDB id 3ZRS) and highlights an important role for H177 at this interface.

3.4.4 S205L increases the cation occupancy in the CTD

Poisson-Boltzmann calculations have predicted a lower electrostatic potential in the cytoplasmic pore for the double-mutant structure compared to the single-

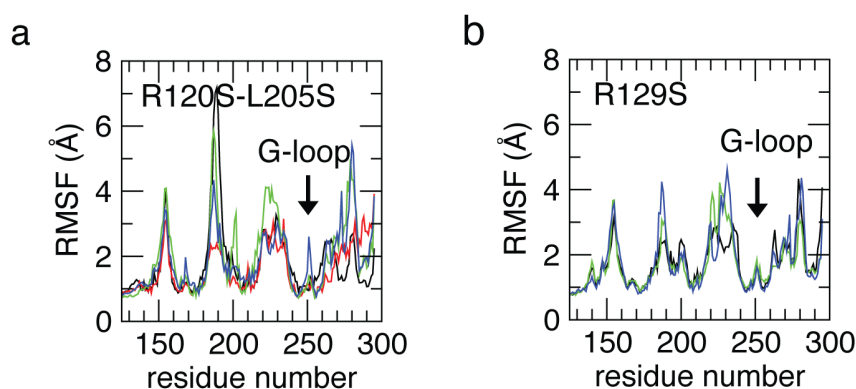


Figure 3.16: RMSF (mobility, coloured by subunit) of C α -atoms in MD simulations of the S129R and the S129R/S205L structures, in which the mutant side chains were replaced with a serine. (a) RMSF of the R129S-L205S and (b) of the the R129S simulation. The G-loop exhibits a higher degree of mobility in the R129S-L205S than in the R129S simulation

mutant structure. Consistent with this prediction, the average cation occupancy in the cytoplasmic pore is higher in the R129S-L205S simulation than in the R129S simulation (Figure 3.14). This is despite the reorientation of the charged side chains of E173 and D175 during the R120S-L205S simulation. Due to this reorientation of these charged side chains, which leads to an electrostatically less attractive potential, the number of Na⁺ ions in the CTD pore gradually diminishes during the R129S-L205S simulation (Figure 3.15a). On the contrary, during the single-mutant simulation, the number of Na⁺ ions remains constantly at low level (Figure 3.15b). Thus, the orientation of E173 and D175 are likely to have a significant effect on the electrical potential of the CTD pore for K⁺ ions.

3.4.5 B-factors inform the network TMD/CTD network

Several domains at the cytoplasmic interface exhibit high degrees of mobility (Figure 3.16). Residues Q252 on the G-loop and R137 on the C-linker at the cyto-

plasmic interface exhibit large B-factors in both the S129R and the S129R/S205L structures and simulations, as does residue E169 on the CD-loop (Figure 3.17a). This is perhaps not surprising, as all these domains were found to link the cytoplasmic assembly to the TM domains as part of an extensive network of interactions. More interestingly, R257 on the carboxyterminal β H strand also exhibits high B-factors and interacts with the backbone of D18 on the N-terminal β Hairpin, which expands the structural interdependency at the cytoplasmic interface [64, 252, 248]. Furthermore, a previously unnoted interaction of R257 with residue E248 on the adjacent G-loop may provide an additional intermolecular pulley, and the E248 backbone carbonyls form salt bridges with the highly mobile R137 on the C-linker (Figure 3.17a).

3.5 Discussion

In this chapter, our knowledge of the conformational landscape of KirBac3.1 was extended. With the help of two new structures, and by using MD simulations and PB calculations, proposed gating transitions in KirBac3.1 could be observed. An overview of all KirBac structures known to date is given in Table 3.1. But what is the significance of these results?

3.5.1 Comments on the new S129R mutant structure

The S129R mutation in the TM2 of KirBac3.1 locks the HBC in an apparent open state. In this open state, TM2 bends at a conserved hinge glycine and rotation of the TM2 opens KirBac3.1 at a secondary gate below the selectivity filter [268]. A network of inter-subunit connections appears to couple the rotation of the

Table 3.1: All known bacterial Kir channel (KirBac) structures to date. Structures of PDB ids 2WLL, 2WLJ, and 2WLK were refined using data deposited in the PDB [64], and are based on structures of PDB ids 1P7B [59], 1XL4 [276] and 1XL6 [277], respectively. § Q170A, † S129R, ‡ S129R-S205L mutants. Q170A and S205L mutations are at the cytoplasmic subunit interfaces, S129R at the helix bundle crossing. Only the S129R and/or S129R-S205L mutants display an open helix bundle crossing. *B. pseudomallei* and *M. magnetotacticum* are of the *Burkholderia* or *Magnetosprillum* genus, respectively.

PDB id	Resolution	Organism	Name	Helix bundle	Ref.
2WLL	3.65 Å	<i>B. pseudomallei</i>	KirBac1.1	closed	[64]
2WLJ	2.60 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLI	3.09 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLO	4.04 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLM	3.61 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLN	3.44 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLH	3.28 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2WLK	2.80 Å	<i>M. magnetotacticum</i>	KirBac3.1	closed	[64]
2X6A	3.10 Å	<i>M. magnetotacticum</i>	§KirBac3.1	closed	[64]
2X6B	3.30 Å	<i>M. magnetotacticum</i>	§KirBac3.1	closed	[64]
2X6C	2.70 Å	<i>M. magnetotacticum</i>	§KirBac3.1	closed	[64]
3ZRS	3.05 Å	<i>M. magnetotacticum</i>	†KirBac3.1	open	[252]
4LP8	2.46 Å	<i>M. magnetotacticum</i>	‡KirBac3.1	open	[249]

cytoplasmic domains to the transmembrane domains.

The KirBac3.1-S129R structure is the first structure of a KirBac channel in an open state. The semi-open PIP₂-bound Kir3.2 (PDB id 3JYC) structure by Whorton *et al.* opens up to 9 Å at the HBC. However, this structure includes the R201A mutation in the C-linker and has only two PIP₂ molecules bound, exposing an asymmetric opening at the HBC. The S129R structure, on the other hand, opens in an iris-like fashion at the HBC, to 17.2 Å, or > 19 Å in R129D simulations (Figure 3.17b). Other K⁺ channels, such as the voltage-gated Kv1.2 channel and a series of KcsA structures are also open at the HBC, but a recent $\beta\gamma$ /Kir3.2 complex only opens up to 6-7 Å (Whorton *et al.*). Thus, unlike for Kv channels, attempts to solve crystals structures of wild-type Kir channels in an open conformation have been unsuccessful. It is perhaps noteworthy that molecular dynamics studies of a Kv channel [278] showed that the lower gate in Kv channels is “frustrated”, i.e. that the Kv channel energetically favours crystallization in an open conformation [279]. Taken together, it appears that KirBac channels prefer to crystallize in the closed state, which probably occupies a minimum energy state.

The regulation of KirBac3.1 by PIP₂ and cholesterol is sensitive to mutations in the CD-loop and the G-loop [280, 270]. Kir channels have been co-crystallised with regulators such as PIP₂ or Na⁺ bound at these loops, and at the C-linker [270, 58, 251]. These interfacial domains play a major role in the mechanisms of Kir channel gating (Chapter 4-5).

Thus, the results of Chapter 3.3 support of a gating model in which coupling of the rotation of the CTD to the TMD seems likely and may lead to opening at the HBC. MD simulations of a CTD mutant structure, it was hoped to gain

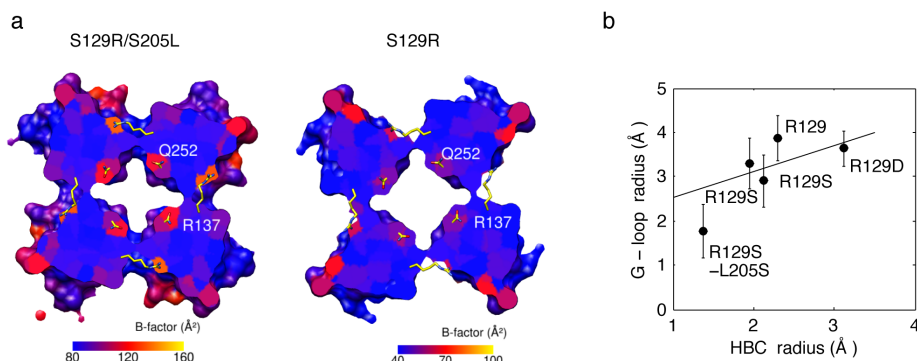


Figure 3.17: (a) B-factors of the KirBac1.3 S129R (left) and S129R-S205L mutant structures. Shown is a cut-away between intracellular assembly and TMD, as seen from the TMD. High B-factors (red to yellow) pinpoint mobile residues Q252 on the G-loop and R137 on the C-linker. (b) Weak correlation between closure of the G-loop and the helix bundle crossing. Each data point corresponds to a 100 ns long MD simulation. The G-loop gate closes most prominently in the R129S-L205S simulation. R129D stabilises the HBC gate in an open conformation, expanding the HBC opening up to 19 $\text{\AA}$ (Y132 C $_{\alpha}$ -C $_{\alpha}$ distance)

insights into the extent of coupling and to obtain qualitative insights into the energy landscape of KirBac3.1 gating and this is addressed below.

3.5.2 Coupling of the G-loop and the HBC

All KirBac structures to date, including cytoplasmic activatory mutants, such as the KirBac3.1-Q170A mutant, exhibit an open G-loop gate and a closed HBC. However, in all simulations, closure of the G-loop occurred together with closure of the HBC. This raises the question of how strongly the CTD, in particular the G-loop gate, is coupled to the HBC gate.

Structurally, the extensive network described in this chapter clearly couples the G-loop, CD-loop, and C-linker to the HBC. To estimate the coupling strength between the CTD and the TMD, the correlation between HBC opening/closing and G-loop opening/closing was calculated for an extended set of simulations

which included an additional R129D simulation (Figure 3.17b). This sampled the conformational landscape for 500 ns in total and thus more efficiently and as when only the R129S and the R129S-L205S simulations would have been used. Unsurprisingly, the correlation between the pore radius of the G-loop and the HBC is weak (correlation coefficient $p = 0.2$) (Figure 3.10). However, it may still be compatible with the notion of moderate coupling, although longer simulations, or approaches that allow more efficient sampling, such as meta-dynamic simulations, would be required to answer this question quantitatively.

Taken together, MD simulations suggests that coupling of the CTD and the TMD involves the slide helices, the HBC, the C-linker, CD-loop, G-loop, and the N-terminus [64, 252, 247] enabling gating at the HBC and the G-loop. This is supported by numerous structural and functional evidence. A particular relation between the conformation of the C-linker and the HBC will be discussed for an inwardly rectifying Kir channel in Chapter 4.

3.5.3 S205L and implications for inward rectification

The simulations suggest that the double mutant S129R/L205S represents an open, higher-energy conformation (Figure 3.13). The MD simulations and PB calculations also highlight an important role for the cytoplasmic inter-subunit interface (Figure 3.12, 3.17). In particular, in all KirBac structures to date, E173 and D175 side chains interact with H177, but in the S205L mutant, a rotameric switch of H177 breaks these interactions and promotes a reorientation of these acidic side chains towards the center of the pore. This affects both the ion occupancy, the electrostatic potential in the CTD, and could have implications for inward rectification in Kir channels.

Clarke *et al.* [64] discuss a model of inward rectification, suggesting that the interface between the intracellular subunits exhibits two distinct states, one “latched”, in which the interactions at the intracellular subunit boundaries are primarily localised towards the membrane, and one “unlatched”, in which a more extensive network of interactions spans across the subunit interface. In their KirBac structures, polyamines were identified between the boundaries of latched, but not unlatched interfaces. It was therefore put forward that, upon activation, unlatching releases bound polyamines into the cytoplasmic pore from which they permeate towards the selectivity filter, to block the pore [64]. In the S129R/S205L structure, the lower delineation of the putative polyamine binding pocket (salt-bridge R202-D197) is strengthened (Figure 3.10), and the positively charged side chains D173 and E175 point away from the interface, towards the pore (Figure 3.11). This extensive array of subunit interactions more closely resembles more the set of interactions found at the interface of the unlatched KirBac3.1 structures.

Therefore, a possible interpretation of the S129R/S205L mutant structure could be that the S205L mutant causes the subunit interfaces to switch into an unlatched configuration which then ejects polyamines into the cytoplasmic pore. By leaving into the pore, polyamines would break the D173-H177-E175 triad, moving the negatively charged side chains D173 and E175 with them towards pore-lining orientations. Upon closure, KirBac3.1 would move from the S129R/S205L conformation towards the latched S129R conformation, and finally rotation of the CTD would lead to closure of the G-loop and the helix bundle crossing. As the simulations here were able to provide a partial reverse gating pathway, there is at least some evidence for this model. However, the

S129R/S205L structure may not represent a fully unlatched conformation and more extensive studies would be necessary to critically assess this interpretation. Nevertheless, this idea can now be used as starting point for further work.

3.5.4 Comments on twisting of the CTD

It has been reported that twisting of the cytoplasmic assembly with respect to the TMD leads to a non-conductive selectivity filter [64]. However, in the new KirBac-S129R structure, Bavro *et al.* showed that the twisted conformation of the CTD exhibits a conductive selectivity filter, directly contradicting the gating model they put forward. Notably, in the majority of MD simulations, the K^+ occupancy in the selectivity filter remained stable at positions S2 and S4 (following the K^+ ion notation of Berneche *et al.* [257]). In a recent structure of Kir3.2 bound to $G\beta\gamma$, a modest rotation (8°) of the CTD with respect to the TMD is observed, apparently lowering the energy barrier between the open and the closed states. In the S129R structure, the CTD rotates by 23° about the pore axis which leads, through coupled interactions between the CTD and the TMD, to an opening at the HBC. However, in the R129S or R129S-L205S simulations, no “untwisting” was observed. This could be because the simulations are too short to overcome the energetic barrier for untwisting, which represents a major conformational change.

Nevertheless, these MD simulations of the new cytoplasmic mutant KirBac3.1 structure expose a possible conformational pathway from the highest-energetic double-mutant (S129R/L250S) conformation via the single-mutant (S129R) conformation towards a fully closed low-energy state thereby expand-

ing the conformational landscape of KirBac3.1 channel gating.

3.6 Summary

Using MD simulations, APBS calculations, and by analysing new X-ray structures, the mechanisms of gating of the prokaryotic Kir channel KirBac3.1 were examined. Kir channels need to open at a constriction called the helix bundle crossing (HBC) in order to be conductive. The new KirBac3.1 S129R and S129R/S205L structures were the first prokaryotic Kir structures solved in an open conformation. These new structures provide an extension to the conformational landscape that is used as a starting point for MD simulations and APBS calculations.

In summary, MD simulations reveal a gating pathway from the high-energy S129R/S205L to the S129R structure. Both structures are open at the helix bundle crossing. Although structurally very similar, the S129R/S205L structure is representative of a higher-energy state. In this pathway, subtle rearrangements in the CTD contribute to a significant change in the electrostatic energy profile in KirBac3.1-S129R/S205L, which would likely alter the rectification properties in eukaryotic Kir channels.

Rotation of the CTD is linked through the C-linker, the CD-loop, and the G-loop to opening at the HBC gate. This is coupled to the reorientation of a negatively charged ring of residues in the intracellular pore. However, eukaryotic Kir channels exhibit a different sequence in the C-linker [11, 134], and recent crystal structures of eukaryotic Kir channels only show modest rotation of the CTD [251]. The possible gating mechanism of the Kir2.2 eukaryotic Kir chan-

nel will be treated in the next chapter, using modelling and MD simulations informed by the most recent structural information for eukaryotic Kir channels [58, 251, 65]).

Chapter 4

Kir gating and lipid interactions

4.1 Introduction

Kir channels gate in response to physiological stimuli (pH, ATP, or receptor stimulation), by opening and closing a constriction at the helix bundle crossing (HBC) (Figure 4.1) [281, 136]. The phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂), which activates all mammalian Kir channels, binds to the C-linker close to the HBC (Figure 4.1) [58], and PIP₂-Kir channel interactions are believed to stabilize the HBC in an open conformation [50, 282]. However, the structural basis of this mechanism of Kir channel activation by PIP₂ remains elusive [283], as all Kir channel crystal structures determined to date are closed at the HBC [58, 251, 284].

In the preceding chapter, computational analyses have shown that mutations in the bacterial Kir channel stabilise the HBC in an apparent open conformation (S129R, S129D, S205L). However, although the crystal structure with the S129R activatory mutation stabilised the HBC gate, in MD simulations of this

4. Kir channel gating and lipid interactions

mutant structure, the HBC closed if the mutation was reverted to the wild-type.

Similarly, a recent study on Kir channels by Meng *et al.* reports simulations in which the HBC opens, but the authors had to resort to introducing a proline mutation in the inner helix [285]. Likewise, MD simulations of wild-type PIP₂-bound Kir channels were unable to report opening of the HBC [286, 287]. In mutant PIP₂-bound Kir channel crystal structures, the HBC gate at most partially opened [251], and this partial opening violated the canonical four-fold symmetry (Kir3.2-R201A).

Thus, a major perturbation, such as a mutation appears to be required to stabilise the HBC in an open conformation, at least in MD simulations or in a crystal structure environment. Consequently, the structural mechanism that stabilises wild-type Kir channels *in vivo* in an open conformation is still unknown, and has not been observed yet, neither *in silico* through simulations, nor in crystal structures. Therefore, in this chapter, without introducing mutations, a structural model of an open wild-type Kir channel is developed which shows that i) the C-linker critically determines the electrostatic profile along the ion permeation pathway, ii) the HBC opens in response to alterations of the C-linker secondary structure, and iii) the PIP₂ binding site at the C-linker can be predicted accurately using a simulation-based approach.

GROMACS

4.1.1 Electrostatic contributions to Kir channel gating

The lipid bilayer composition critically determines ion channel activity. PIP₂ accounts for 2% of phospholipids in the mammalian plasma membrane inner leaflet [288], and directly activates all Kir channels, as well as many other ion

4. Kir channel gating and lipid interactions

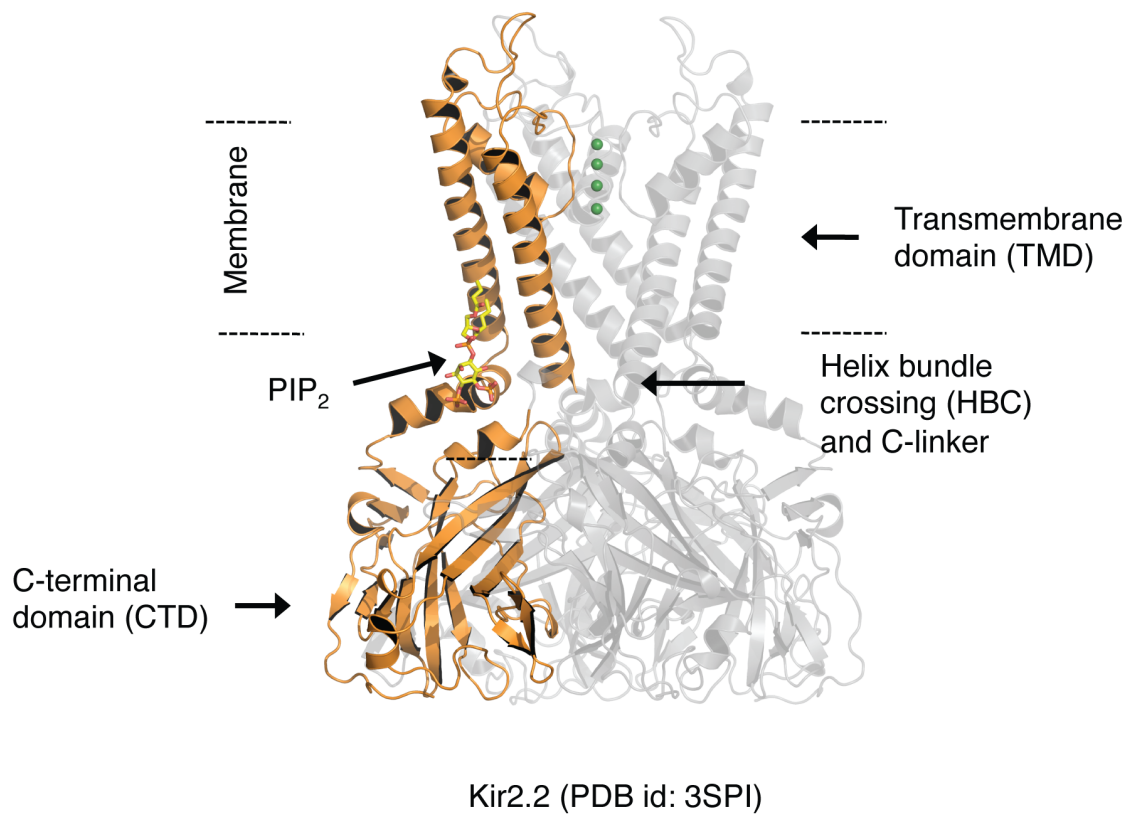


Figure 4.1: Overall tetrameric structure of Kir2.2 (PDB id: 3SPI), with one subunit highlighted in orange and ions in the selectivity filter shown as green spheres. The 3SPI structure was co-crystallized with PIP₂ (shown as yellow sticks). Arrows indicate PIP₂, the C-terminal domain (CTD), helix bundle crossing (HBC), C-linker, and transmembrane domain (TMD).

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channels and transporters [289]. Kir channels exhibit varying sensitivity to activation by different phosphoinositol (PIP) isoforms [290, 291]. Whilst members of the Kir1 and Kir2 channel family are particularly sensitive to activation by PIP₂, Kir3 channels and, in particular, Kir6.2, are activated by PI(3,5)P, and PI(3,4,5)P, PI(4), PI(5) as well [291, 292]. This PIP-activation pattern of Kir channels has been studied extensively in cell-based and in liposomal patch-clamp and ⁸⁶Rb⁺ flux experiments, as well as in binding assays [140]. Therefore, molecular dynamics simulations may be compared to a range of functional data, as well as to the X-ray crystal structures of Kir channels [58, 251, 284].

Critically, although Kir channel crystal structures recently reported the binding site of PIP₂, the exact molecular mechanism by which PIP₂ activates Kir channels is still subject of considerable speculation [58, 251]. Poisson-Boltzmann calculations in the preceding chapter showed that small changes in the cytoplasmic domain (CTD) significantly altered the electrostatic barrier for K⁺ ion permeation. Therefore, one may speculate that a small displacement of a charged amino acid, for example on the C-linker, may lead to activation through the removal of electrostatic barriers, a mechanism which has also been implicated in other K⁺ channels [89]. Given that the highly negatively charged head group of the activatory phospholipid PIP₂ binds to basic residues on the flexible C-linker near the HBC (Figure 4.1), the variation of the energetic profile at the HBC seems worth exploring.

The first part of this chapter (Section 4.3) therefore studies this relationship between the C-linker and the electrostatic energy profile by comparing two recent Kir2.2 crystal structures by Hansen *et al.* [58] and by neutralising a basic PIP₂-interacting residue on the C-linker. Based on the observation that the C-

4. Kir channel gating and lipid interactions

linker of phospholipid-bound partially open Kir channel structures is partially helical [58, 251, 284], the C-linker will be modelled in a fully helical conformation, assuming that the resulting model will be fully open. Interestingly, a fully open model will be obtained, the first wild-type model of a Kir channel with an open HBC gate.

4.1.2 Previous PIP₂ binding studies

Lipid interactions with membrane proteins are an important component of many cell signaling pathways. The secondary messenger PIP₂ not only activates Kir channels but also localises proteins to membranes, and regulates the activity of many different classes of ion channels [293] and transporters.

Previous computational studies of PIP₂-Kir channel binding included both conventional docking and molecular dynamics approaches [294, 162, 286]. However, these previous studies all employed homology models based upon the structure of a chimera between a eukaryotic and prokaryotic Kir channel in which several of the residues proposed to interact with PIP₂ were not fully resolved (PDB id 2QKS). Furthermore, this chimeric Kir/KirBac channel exhibits a complex functional interaction with PIP₂ because it is known that PIP₂ inhibits prokaryotic Kir channels [289, 280]. Consequently, the accuracy of the binding site predicted in these original simulations remains uncertain.

The second part of this chapter (Section 4.4), in addition to discussing mechanisms of Kir channel gating, takes advantage of the recently solved PIP₂-bound X-ray crystal structure of the eukaryotic Kir2.2 channel [58], to validate a simulation-based method for the prediction of PIP₂-binding to an ion channel [162].

4.1.3 Aims

While it is not the aim to assert that Kir channels open as suggested by the open-HBC Kir channel model, it is hoped that the new model, together with the validated simulation-based prediction method for PIP₂-channel interactions, will give new insights into the role of PIP₂ in Kir channel gating and help to further interpret a comprehensive body of existing functional data. Moreover, Chapter 4 also aims to demonstrate how computational tools can be used to separate the study of PIP₂-binding to an ion channel from the downstream gating transitions following PIP₂ binding. This separation is inherently difficult to achieve in experiments [295, 296] and has only very recently been approached using biochemical arrays of PIP₂ binding to Kir2.1 channels [292].

4.2 Methods

4.2.1 Analysis of structures and modelling

The PIP₂-bound Kir2.2 (PDB id: 3SPI) crystal structure was compared with the Kir2.2 structure bound to the anionic phospholipid pyrophosphatidic acid (PPA, PDB id: 3SPC) using Pymol and VMD [35, 36]. *In-silico* mutations R186A and R186D were introduced using Pymol. HOLE was used to calculate the pore radius profiles [261].

Missing loops on the N-terminus were modelled using MODELLER. The N-terminus primarily regulates channel gating transitions rather than binding of phosphoinositols itself [292]. Therefore the key interactions that govern binding of PIP₂ are independent from the conformation of the N-terminal model and

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the model with the lowest MODELLER score was used for MD simulations [225, 297].

The model of Kir2.2 with an open helix bundle crossing (HBC) was created using MODELLER and the simulated annealing method, employing the 3SPI structure as a template. Helical constraints were applied to residues comprising the C-terminal end of TM2 and the C-linker. All other residues were masked during the modelling simulations so that they kept their crystallographic conformation. Models with a closed helix bundle were generated as controls, in the same way as open models, but without application of restraints, except for symmetry restraints, which were imposed on all generated open and closed models.

4.2.2 Calculations of electrostatic energy profiles

Electrostatic energy profiles of the Kir2.2 3SPI and 3SPC wild-type and R186A or R186D mutant structures were calculated. The method was employed as described by Amiri *et al.* [300]. The K^+ ion was placed in successive positions of 1 Å spacings along the pore axis, in the interval ranging from the CD-loop in the cytoplasmic domain ($z = -15$ Å) to the HBC ($z = 25$ Å) at the cytoplasm-membrane interface. Sample points were obtained from HOLE [261]. Hydrogens were added to the structure using PDB2PQR, which predicted charges and radii for use with APBS package to calculate the Born energy of the K^+ ion at each sampling point along the pore axis [256, 235]. The K^+ ion had a charge of $+1e$ and a radius of 1.96 Å [232]. For each individual sample point a calculation was performed in a cube of dimensions $97 \times 97 \times 97$ Å³ comprising $200 \times 200 \times 200$ grid points. Fine grid focusing was used as described in Amiri

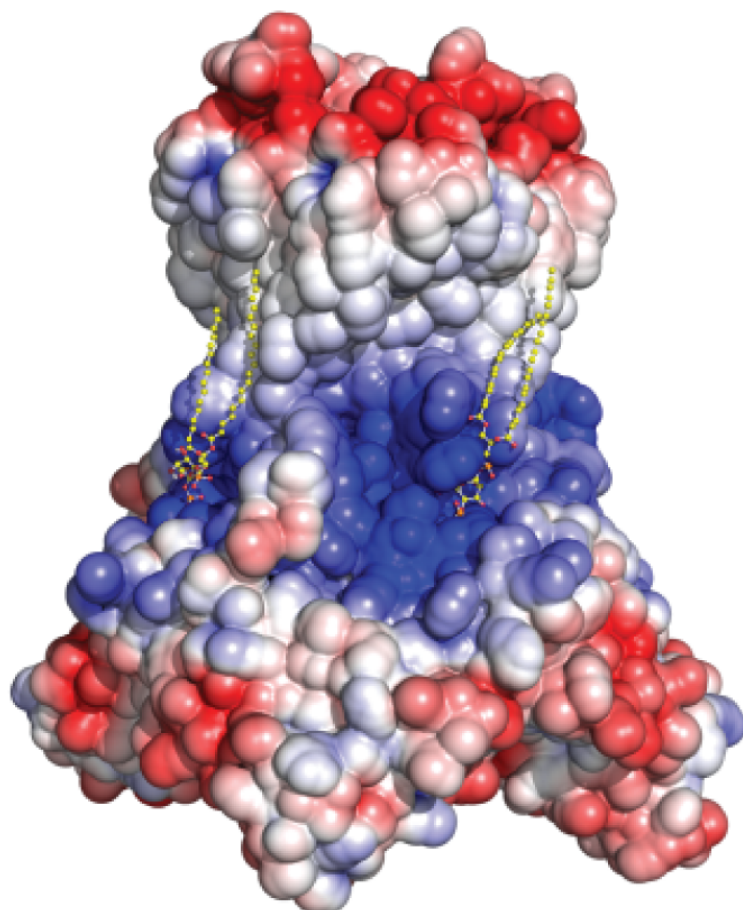


Figure 4.2: Electrostatic potential. Snapshot from a 3SPI multiscale prediction, after 0.1 μ s of atomistic simulation, coloured by electrostatic potential. PDB2PQR [256] and PROPKA [298] were used to assign charges, radii, and protonation states. APBS Tools [299] and Pymol were used to map the electrostatic potential, coloured from 4 kT/e (red) to +4 kT/e (blue), onto the solvent-accessible surface. PIP₂ is shown in a ball and stick representation. The binding site of the PIP₂ head group coincides with areas of positive electrostatic potential.

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et al. in a cube with dimensions $40 \times 40 \times 40 \text{ \AA}^3$. Energies were obtained by solving the Poisson-Boltzmann equation at $T = 298.15 \text{ K}$, at an ionic strength of 0.1 M NaCl . The protein was assigned a dielectric constant $\epsilon = 10$, and the surrounding solvent a dielectricity of $\epsilon = 80$.

In addition, the electrostatic energy profile of the 3SPI structure was calculated with PIP₂ coordinates as found in the crystal structure. Charges and radii for PIP₂ were assigned using AUTODOCK [241]. The PIP₂ head group was replaced with the head group of pyrophosphatidic acid, as obtained from the 3SPC structure, and the electrostatic energy profile of this PPA-bound structure was compared to the PIP₂-bound structure. The total charges of ligands were $-5e$ (PIP₂) and $-3e$ (PPA) [301, 302].

4.2.3 Multi-scale simulations

The binding site of phosphatidylinositol 4,5-bisphosphate (PIP₂) on the inwardly-rectifying potassium channel Kir2.2 was predicted using a multi-scale simulation approach as described previously [162]. The setup and the analysis of the CG and AT molecular dynamics simulations are shown in Figure 4.3. The electrostatic potential is mapped on the binding site of PIP₂ (Figure 4.2), and the CG force field parameters used for PIP₂ (Figures 4.4 and 4.5) are reported.

The PIP₂ binding site was predicted using the multi-scale approach [162], consisting of CG simulations, which were then followed by AT simulations [219]. Two crystal structures of Kir2.2, wt-Kir2.2 bound to a short-chain (scPIP₂) derivative of PIP₂ (PDB id 3SPI), and wt-Kir2.2 bound to an anionic lipid (PDB id 3SPC), were used as starting structures of the multi-scale simulations. In order to prepare the starting structures of the multi-scale simulations, the scPIP₂

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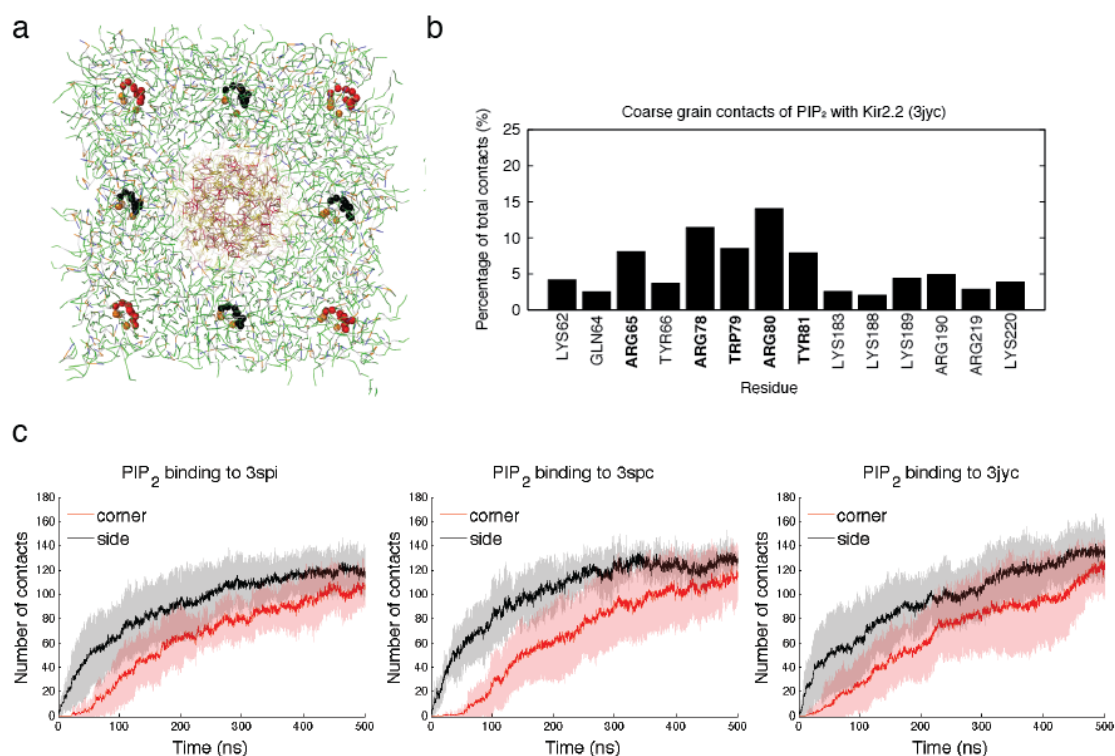


Figure 4.3: Coarse-grained PIP₂-binding. **(a)** Setup of a CG binding simulation. Before the start of the simulation ($t = 0 \mu s$), PIP₂ is added to the lower leaflet of the lipid bilayer, in two different starting locations: either to the corners of the simulation box (red CG PIP₂ particles), or to the sides of the simulation box (black CG PIP₂ particles). **(b)** Residues making contacts with PIP₂ head groups in CG simulations of the lipid-unbound state (PDB id 3JYC), residues in bold account for $> 5\%$ of the total contacts. **(c)** Average number of contacts between the PIP₂ head groups and Kir2.2 in CG simulations for each starting configuration [red and black, corresponding to colours used in **(a)** for red and black CG PIP₂ particles], and for each input structure of PDB ids 3SPI, 3SPC and 3JYC.).

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and the anionic lipid were removed, and the structures embedded into a 1-palmitoyl-2-oleoylphosphocholine (POPC) lipid bilayer, with four PIP₂ molecules added to the intracellular leaflet (Figure 4.3a). For each starting structure, CG simulations ($24 \times 0.5 \mu\text{s}$) were performed using the Martini parameters, showing binding of PIP₂ to the protein (Figure 4.3b). Snapshots were taken at $0.5 \mu\text{s}$ from CG simulations, and converted into atomistic systems. The coarse-grained protein was converted into an atomistic representation using the align option [219], by realignment of the original atomistic PDB coordinates that were used as starting structures of the multi-scale simulations. GROMOS96, Berger lipid parameters, and the SPC water model were used for AT simulations ($2 \times 0.1 \mu\text{s}$ / starting structure) [197, 196, 303, 185]. The systems were prepared for AT simulations by placing potassium ions at position S2 and S4 of the selectivity filter. The cavity below the selectivity filter was solvated using VOIDOO [258]. Before the start of the AT simulations, systems were energy-minimized using 5000 iterations of steepest descent, followed by a 1 ns AT simulation with positional restraints of 1000 kJ/mol/nm^2 applied in each direction to the proteins heavy atoms. In addition to 3SPC and 3SPI multi-scale simulations, CG simulations of the 3SPC structure of wt-Kir2.2 (PDB id 3JYC), suggest that PIP₂ may be able to bind to Kir2.2 in the 3SPC-state as well. (Figure 4.3c).

Basic residues predicted to bind PIP₂ coincide with regions of positive electrostatic potential on Kir2.2 (Figure 4.2). To test for a possible influence of the lipid bilayer composition on PIP₂ binding, CG simulations were repeated with half of the inner leaflet POPC lipids randomly replaced by the anionic lipid palmitoyl-oleoyl phosphatidylserine (POPS).

The reference simulations ($2 \times 0.1 \mu\text{s}$) were performed with PIP₂ placed

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```

[moleculetype]
; molname      nrexcl
PIP2 1

[atoms]
; id  type  resnr  residu  atom  cgnr  charge
1  Qa  1  PIP2  PO1  1  -2.0
2  Qa  1  PIP2  PO2  2  -2.0
3  SP1  1  PIP2  RP1  3  0
4  SP1  1  PIP2  RP2  4  0
5  SP1  1  PIP2  RP3  5  0
6  Qa  1  PIP2  PO3  6  -1.0
7  Na  1  PIP2  GL1  7  0
8  Na  1  PIP2  GL2  8  0
9  C1  1  PIP2  C1A  9  0
10 C1  1  PIP2  C2A  10  0
11 C1  1  PIP2  C3A  11  0
12 C1  1  PIP2  C4A  12  0
13 C1  1  PIP2  C1B  13  0
14 C1  1  PIP2  C2B  14  0
15 C1  1  PIP2  C3B  15  0
16 C1  1  PIP2  C4B  16  0
17 C1  1  PIP2  C5B  17  0

[angles]
; i j k      funct  angle  force.c.
1 3 6      2    180.0  45.0
2 4 6      2    120.0  45.0
1 5 6      2    180.0  45.0
2 5 6      2    180.0  45.0
3 4 5      2    120.0  25.0
4 5 3      2    120.0  25.0
5 6 7      2    180.0  25.0
6 7 8      2    120.0  25.0
6 7 13     2    180.0  25.0
7 8 9      2    180.0  25.0
8 9 10     2    180.0  25.0
9 10 11     2    180.0  25.0
10 11 12    2    180.0  25.0
7 13 14     2    180.0  25.0
13 14 15    2    180.0  25.0
14 15 16    2    180.0  25.0
15 16 17    2    180.0  25.0

[bonds]
; i j  funct  length  force.c.
1 3 1    0.30  5000
2 3 1    0.34  5000
2 4 1    0.34  5000
3 4 1    0.30  20000
3 5 1    0.30  20000
4 5 1    0.30  20000
5 6 1    0.30  5000

```

Figure 4.4: Coarse-grained force field parameters for PIP₂. The parameter format follows the standard GROMACS parameter format (see GROMACS user manual [187], version 4, Section 5.7, <ftp://ftp.gromacs.org/pub/manual/manual-4.5.6.pdf>)

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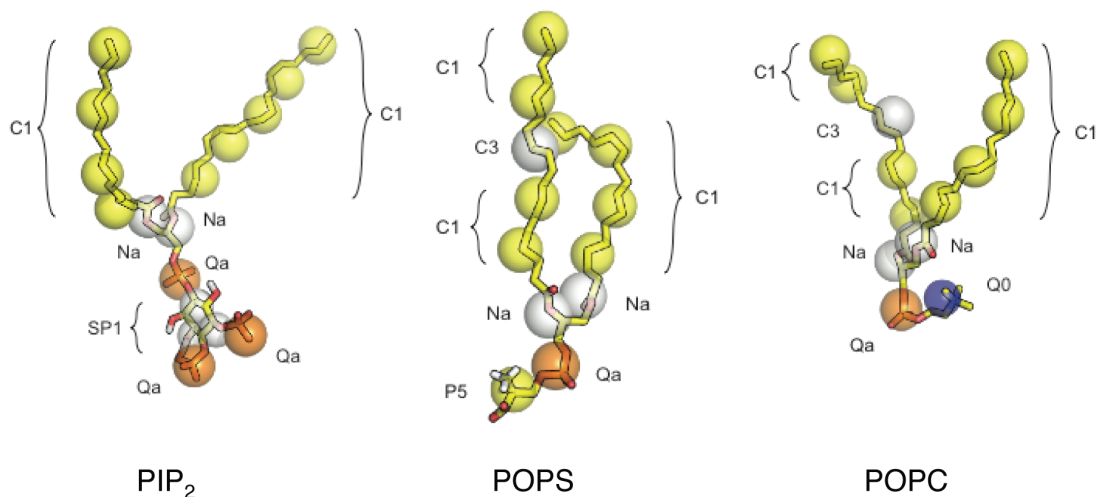


Figure 4.5: MARTINI particle types used for coarse-grained simulations (also see Table 1) [213]. The MARTINI 2.1 force field parameters were used for POPS and POPC lipids.

in the location in which scPIP₂ was observed in the crystal structure (PDB id 3SPI). In order to set up the reference simulations, a 0.1 μ s CG self-assembly simulation of 3SPI in POPC was performed, followed by conversion into an atomistic system (using the align option). The head group of PIP₂ was aligned to scPIP₂ and any clashing lipids were removed. After energy minimization of the system, positional restraints of 1000 kJ/mol/nm² were applied in each direction for 1 ns to the proteins heavy atoms and to the head group atoms C₁-C₆ of PIP₂ followed by 0.1 μ s of unrestrained AT simulation. This ensured that, at the start of the unrestrained 0.1 μ s reference simulations, the coordinates of PIP₂ coincided with the coordinates of scPIP₂ from the crystal structure. The reference simulations were repeated with PIP₂ removed from the protein before the start of the simulation (equilibration, 2 x 0.1 μ s, Figure 4.6), to test whether

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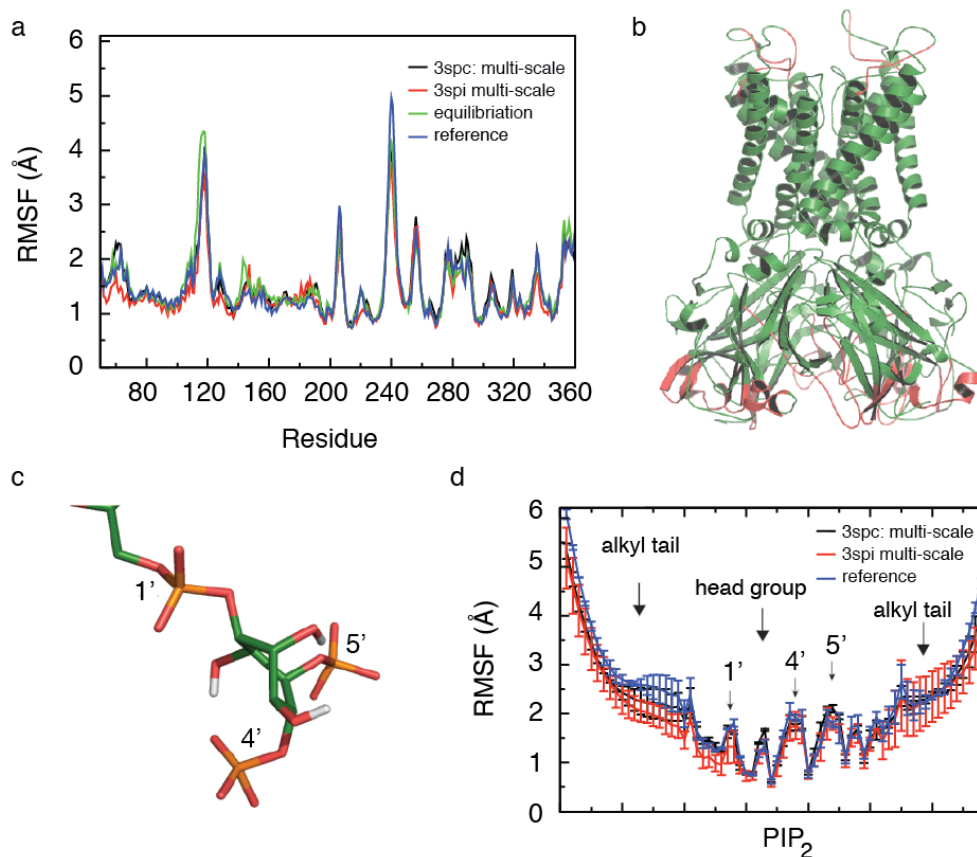


Figure 4.6: RMSF analysis of the PIP₂-binding site. (a) Average root mean square fluctuations (RMSF) of C_{α} atoms, calculated for 3SPC and 3SPI multi-scale simulations ($2 \times 0.1 \mu\text{s}$ / input structure), reference simulations ($2 \times 0.1 \mu\text{s}$), and equilibration simulations (3spi, PIP₂ removed, $2 \times 0.1 \mu\text{s}$). (b) Residues with average RMSF $> 2.5 \text{ \AA}$ (red) mapped on a snapshot of an atomistic simulation. (c) Nomenclature used in (d). (d) Average RMSF of PIP₂. Phosphates (1', 4' and 5') with adjacent oxygen atoms are highlighted by small arrows.

the lipid-bound structure was able to equilibrate into a structure similar to the Kir2.2 3SPC-state, and RMSF values computed using GROMACS4.5.5 (Figure 4.6) [185, 184]. Run-time parameters for MD simulations were set as described in the preceding chapter.

4.3 Kir2.2 channel gating

Crystal structures of eukaryotic Kir channels, first published in the year 2011, offer the advantage that Kir channel gating can be studied without the need to build homology models based on prokaryotic templates [65]. Also, the activatory lipid PIP₂ binds to residues on the C-linker connecting the TMD and CTD, which do not exist in prokaryotic Kir channels. The computational study of Kir channels therefore benefited from the eukaryotic Kir2.2 and Kir3.2 structures [65, 58, 251, 284].

4.3.1 Comparison of closed state structures

The most recently determined structures of Kir2.2 (Figure 4.7a) are either co-crystallised with PIP₂ (PDB id: 3SPI), or with a non-specific phospholipid (PDB id: 3SPC) instead of PIP₂. Comparison of these two structures shows that the 3SPC structure is closed at the HBC, with a steric constriction formed by the four side chains of M181 [58], Figure 4.7b), while the 3SPI structure is possibly just beginning to open. A closer comparison of these two structures also reveals an additional conformational change in the C-linker (Figure 4.7c). In the Kir2.2 3SPI (PIP₂-bound) structure, the side chain of R186, which is relatively well conserved among Kir channels, is turned by 180° relative to its conformation in

4. Kir channel gating and lipid interactions

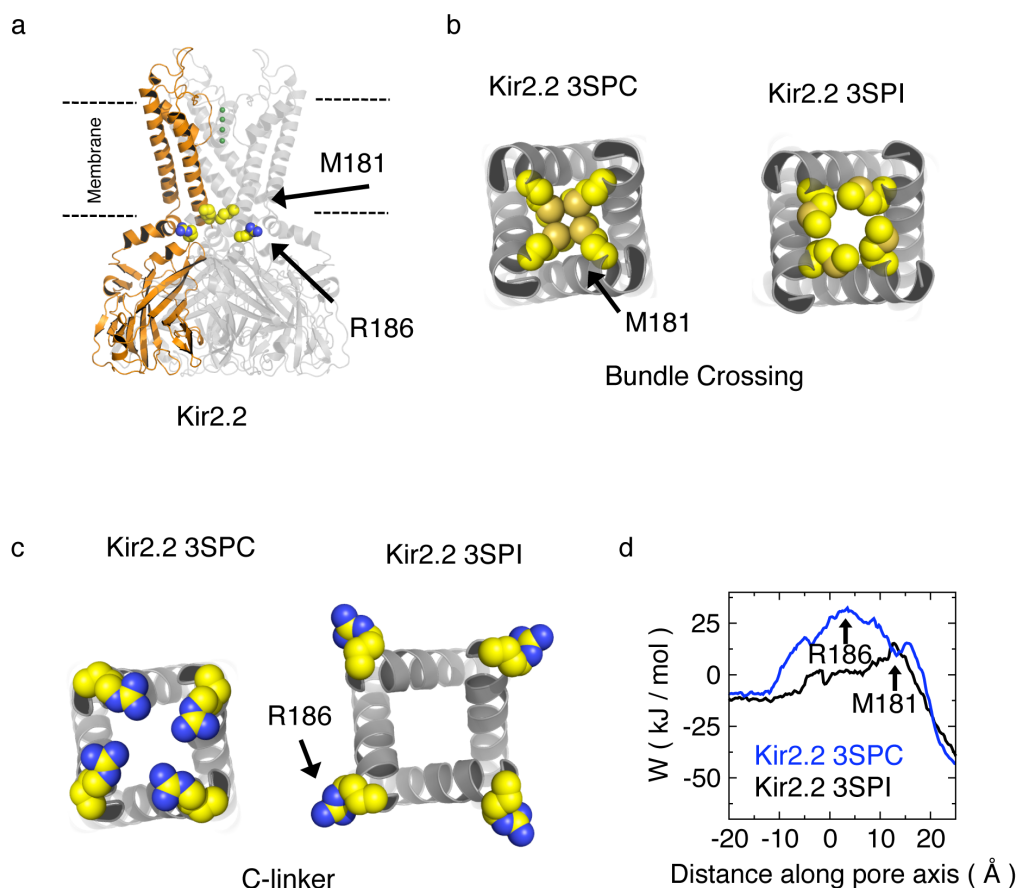


Figure 4.7: Structure of the Kir2.2 potassium channel and comparison of the electrostatic potential along the pore axis of the Kir2.2 3SPC (PDB id: 3SPC) and 3SPI (PDB id: 3SPI) wild-type structures. (a) Overall structure of Kir2.2, with one subunit shown in orange for clarity. Ions in the selectivity filter are shown in green. Residues M181, which forms the primary gate at the helix bundle crossing (HBC), and R186 on the C-linker, are shown in yellow. (b) Bottom up view of the HBC gate in Kir2.2 3SPC (co-crystallized with a non-specific lipid) and Kir2.2 3SPI [co-crystallized with phosphatidylinositol (4,5)-bisphosphate (PIP₂)] structures. The 3SPC structure is closed at the HBC, while the 3SPI structure is possibly just beginning to open. (c) Bottom-up view of R186 in Kir2.2 3SPC and 3SPI structures. (d) Electrostatic pore profile of the Kir2.2 3SPC and Kir2.2 3SPI structures. Barriers for ion permeation at the HBC and on the C-linker are formed by residues M181 and R186. The 2Fo-Fc maps display full connectivity for the R186 side chain at 1.5 σ (3SPI) or 1.1 σ (3SPC).

4. Kir channel gating and lipid interactions

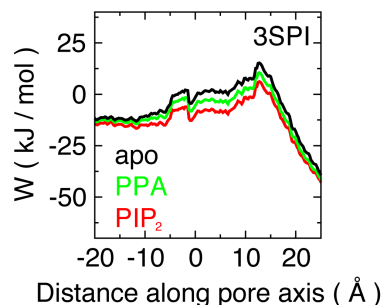


Figure 4.8: Electrostatic energy profile of the 3SPI structure in the lipid-unbound, PPA-bound, or PIP₂-bound configuration. For the PPA configuration, the pyrophosphate head group from the 3SPC structure replaced the PIP₂ head group of the 3SPI crystal structure

the 3SPC (PPA-bound) structure, pointing away from the ion permeation pathway. Since R186 forms part of the binding site of the PIP₂ ligand, this suggests that this conformational change in the C-linker arises from the binding of PIP₂, which would cause the 180° re-orientation of the R186 side chain (Figure 4.7c).

Importantly, this re-orientation of R186 also has a significant effect on the electrostatic energy profile (Figure 4.7d). The electrostatic Poisson-Boltzmann profile of a K⁺ ion in the cytoplasmic pore of the 3SPC structure exhibits a markedly higher barrier for K⁺ ion permeation than the 3SPI structure. The maximum barrier height is formed by R186 in the 3SPC structure. Instead, in the 3SPI structure, with R186 pointing away from the pore, the maximum barrier height results from the constriction of the M181 side chains at the HBC (Figure 4.7b).

This electrostatic energy profile provides a first approximation of a free energy profile of K⁺ ions moving along the pore axis of an ion channel [304, 300]. In general, only energy barriers > 2kT (5 kJ/mol) are considered significant, as

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energies below that level can be easily overcome by small thermal fluctuations. If PIP₂ is explicitly considered in the calculation of the energy profile of the 3SPI structure, the cytoplasmic pore becomes nearly permeable (Figure 4.8). However, the permeation pathway is still sterically impeded by the narrow constriction at the HBC gate (pore radius <2 Å at the HBC), and the effective energetic barrier for permeation at the position of the HBC is determined by the energy of desolvation needed for a K⁺ ion to pass the hydrophobic gate at M181. Desolvation effects are generally underestimated by electrostatic energy profiles [255].

Overall, comparison of the 3SPC and the 3SPI structures suggests that i) the C-linker and the hydrophobic constriction at the HBC present a significant barrier to K⁺ ion permeation and ii) the electrostatic component of this barrier arises primarily from the C-linker and iii) the electrostatic component depends critically on the precise conformation of the C-linker.

4.3.2 Mutation of closed state pores

To further investigate the relation between the C-linker and the electrostatic energy profiles, the effect of *in-silico* mutations on the electrostatic energy profile and on the pore radius was studied. This approach has previously been applied to Kir channels [254, 305] and model pores [268, 306].

The electrostatic profile of Kir channels is sensitive to bound ligands and to mutations of pore-lining residues. Robertson *et al.* suggested that the Kir2.1 residue corresponding to R186 contributes > 4 kJ/mol energy to the interaction of a K⁺ ion along the pore of Kir2.1 homology models [254]. Furthermore, the Kir2.1-K185Q mutant, while retaining sensitivity to PIP activation in liposomes,

4. Kir channel gating and lipid interactions

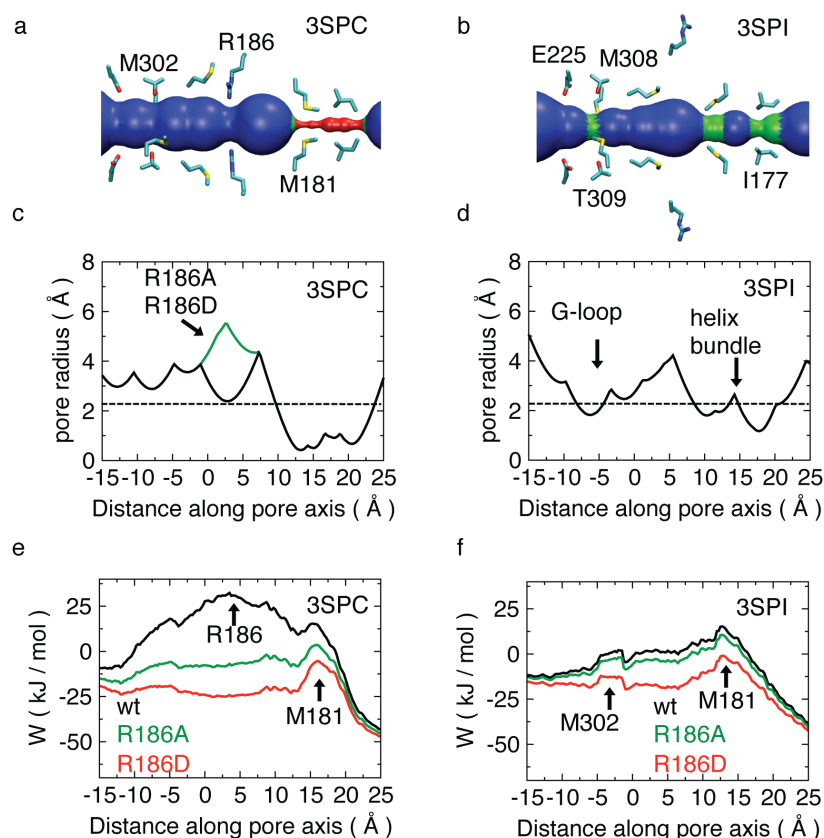


Figure 4.9: Effect of mutations on the pore radius r and the electrostatic energy profiles of Kir2.2. The electrostatic energy profiles for K^+ permeation along the pore axis were calculated using APBS. **(a)** Visualisation of the cytoplasmic cavity of the 3SPC structure (blue, $r > 2.2$ Å), and of the constriction at the helix bundle crossing (red, $r < 1.15$ Å). A pore radius of 2.2 Å is the minimum required for a solvated K^+ ion to pass. Side chains of pore-lining residues are shown as sticks, with labels attached to residues that contribute significantly to the hydrophobic or electrostatic barrier for K^+ ion permeation. **(b)** Analogous visualisation for the 3SPI structure with labels attached to those pore-lining residues which have only an ancillary effect on the electrostatic pore profile. The cytoplasmic loop lined by T309 and the helix bundle crossing are only permeable for desolvated K^+ ions (1.15 Å $< r < 2.2$ Å). **(c)** Pore radius of the 3SPC structure versus distance along the pore axis. The pore radius of the R186A mutant structure is shown in green. **(d)** Pore radius of the 3SPI structure. Constrictions formed by T309 (G-loop) and by M181 (helix bundle) are marked. **(e-f)** Impact of the R186A and R186D mutants on the electrostatic energy profile. Note that the energy profile of the 3SPC-R186A structure **(e)** is almost identical to the energy profile of the wild-type 3SPI structure **(f)**.

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exhibits low specificity for activation by PIP₂ [291]. Therefore, to test whether the different electrostatic barrier of the Kir2.2 3SPI and 3SPC structure is a direct consequence of the side chain reorientation of R186, the effect of the R186A and R186D mutations on the pore radius and the electrostatic profile was studied.

The cytoplasmic cavity, the radius and the electrostatic energy profile of the R186A, R186D, and wild-type 3SPC and 3SPI structures are shown in Figure 4.9. Both the R186A and R186D mutations increased the pore radius of the 3SPC structure by 3 Å at the C-linker (4.9a, c). No noticeable effect of either mutant on the pore radius of the 3SPI structure was observed (Figure 4.9b, d). Interestingly, both mutants had a major effect on the electrostatic energy profile of the 3SPC structure (Figure 4.9e), but only the charge reversal of mutant R186D had a noticeable effect on the 3SPI structure (Figure 4.9f), suggesting that the reorientation of the R186 side chain compensates for the R186A charge neutralisation.

Amongst the pore-lining residues (E225, M302, M309, T309, R186, M181, and I177), major constrictions ($r < 2.2$ Å) correspond to M302 at the G-loop and M181 and I177 at the helix bundle. Compared to the R186-mutation, these hydrophobic residues had a minor effect on the electrostatic energy profile. Their role is probably to sterically impede the ion conduction as several gating models and MD-simulations of the G-loop and the HBC suggest. At small steric constrictions ($r < 1.6$ Å), van der Waals forces become so large that the electrostatic energy profile can no longer approximate the free energy profile of K⁺ ions moving through the pore [255].

Collectively, these results suggest that R186 may be a determinant of the electrostatic barrier to K⁺ ion permeation. The energy profile of the R186A-

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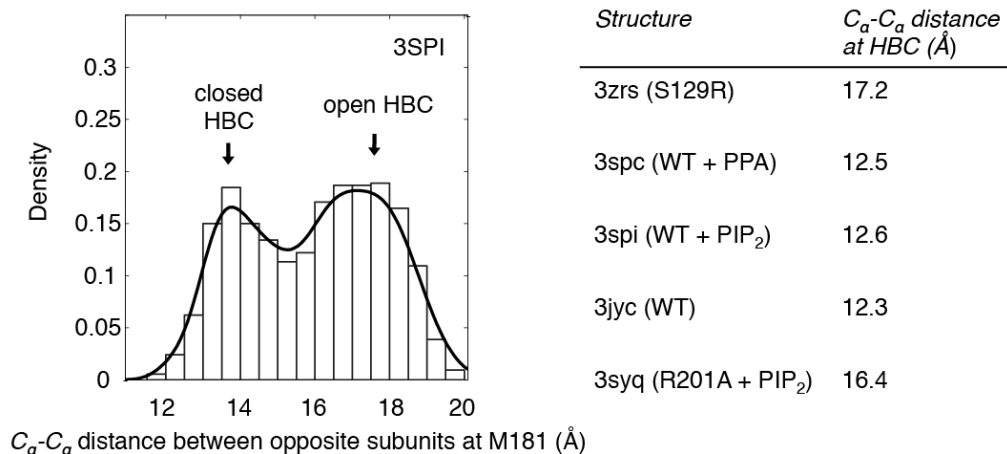


Figure 4.10: Gating of Kir2.2 at the helix bundle crossing (HBC). Distribution of C_{α} - C_{α} distances of the gating residue M181 at the HBC, averaged over $6 \times 0.1 \mu\text{s}$ atomistic simulations of the 3SPI structure embedded in a POPC bilayer, with PIP₂ removed from the 3SPI structure.

3SPC structure is strikingly similar to the energy profile of the wild type 3SPI structure, suggesting that the effect of PIP₂ binding on the electrostatic profile is a direct consequence of the reorientation of the R186 side chain.

4.3.3 Opening of the helix bundle crossing

No opening of the constriction at the HBC could be seen in MD simulations of Kir2.2 [286, 287]. To confirm this result, PIP₂ was initially removed from the 3SPI structure, and the structure embedded in a POPC bilayer. This system was then subjected to atomistic simulations ($6 \times 0.1 \mu\text{s}$). For each simulation, the opening at the HBC was measured as the C_{α} - C_{α} distance between M181, the residue forming the narrow constriction at the HBC. Surprisingly, the average distribution of the M181- C_{α} - C_{α} -distances appeared very broad, and somewhat bimodal. Notably, the 17-Å maximum seen in the distribution corresponded

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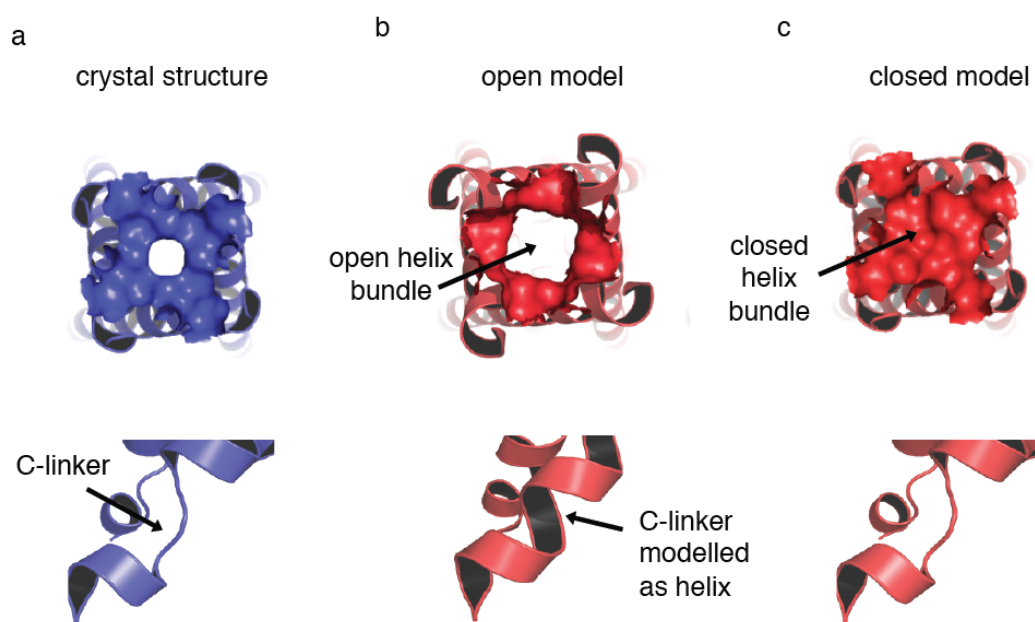


Figure 4.11: Modelling Kir2.2 with an open helix bundle crossing (HBC). Bottom up view (top) of the HBC and side view (bottom) of the C-linker. **(a)** The HBC gate in the Kir2.2 3SPI structure (PDB id: 3spi) is possibly just beginning to open and the C-linker forms a partial helix. **(b)** Open Kir2.2 model based on the 3SPI structure. Note the C-linker, which is modelled as a continuous helix. **(c)** Closed Kir2.2 model based on the 3SPI structure, without helical restraints applied.

well to the 17-Å opening seen in the crystal structure of the open KirBac3.1-S129R mutant (Figure 4.10). The simulations were repeated ($2 \times 0.1 \mu\text{s}$) with the PIP₂-bound 3SPI structure, but this only had a weak influence on the distance distribution of M181-C_α-C_α atoms at the HBC. Although this is the first molecular view of Kir channel gating at the HBC in simulations of wild-type channels, the distribution of possible degrees of opening at the HBC is diffuse and the opening at the HBC of the 17-Å PIP₂-bound state seems not significantly more stable than in the lipid-unbound state.

To probe a possible open state with a significantly higher conformational stability at the HBC, MODELLER [225] was used to introduce plausible structural

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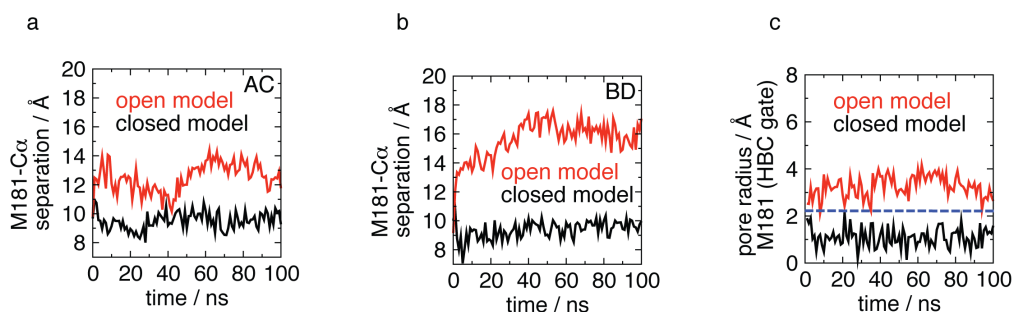


Figure 4.12: Stability of open and closed Kir2.2 models in unrestrained 100 ns long molecular dynamics simulations. **(a)** The pore radius, calculated with HOLE [261], for the HBC (M181). The HBC gate remains permeable for K^+ ions in the open model but remains closed in the closed model. The radius of a hydrated K^+ ion is indicated. **(b-c)** Separation of M181-C α atoms located on opposite subunits at the HBC. The initial M181-C α separation increases by up to 5 Å in the open model and decreases by 3 Å - 4 Å in the closed model. The maximum M181-C α separation observed in the open model (17 Å) is identical to the separation found at the HBC in the semi-open PIP₂-bound Kir3.2-R201A structure [251] and in the apparent-open KirBac3.1-S129R structure that was studied in the preceding chapter [252].

changes, without introducing mutated amino acids. The recent Kir3.2 structure with bound phospholipids, although only beginning to open at the HBC (15 Å), possesses a partially helical C-linker. To test whether helicity of the C-linker is conducive to opening at the HBC, a Kir2.2 model based on the 3SPI structure (PIP₂-bound) was built using helical constraints. Interestingly, compared with the 3SPI crystal structure (Figure 4.11a), the resulting models ($n = 12$) were open at the HBC (Figure 4.11b). Conversely, if the model was built without helical constraints, the HBC was closed (Figure 4.11c). During a 0.1 μ s long atomistic simulation, the distance of M181-C α atoms of each pair of subunits increased from 12.6 Å to 17 Å (Figure 4.12a, b). This extent of opening at the HBC corresponds to the apparently open HBC of the homologous S129R mutant structure (Y132 C α -C α -distance 17 Å). Here however, no additional constriction such as in the S129R structure is blocking the pore. The pore radius of the open model

remains stable at $3 \text{ \AA} \pm 0.3 \text{ \AA}$ over $0.1 \text{ }\mu\text{s}$ (Figure 4.12c). On the other hand, if the model was built without using helical constraints, the M181-C $_{\alpha}$ -C $_{\alpha}$ distances between each pair of subunits decreased to $9 \text{ \AA}$, and the pore radius remained well below that of a hydrated K $^{+}$ ion (Figure 4.12c).

4.4 Multi-scale simulations of PIP $_2$ binding

Using the multi-scale simulation method [162], this section takes advantage of the crystal structures of the eukaryotic Kir2.2 channel in the PIP $_2$ -bound conformation, and of a Kir2.2 structure bound to the anionic non-specific lipid PPA to predict lipid/ion channel interactions, in order to validate this multi-scale simulation method for the purpose of the prediction of a PIP $_2$ binding site.

This multi-scale simulation method employs coarse-grained (CG) simulations to predict initial PIP $_2$ -binding events which are then refined by subsequent atomistic (AT) simulations. In order to validate this prediction, the simulations are then compared to the crystal structures of Kir2.2 with either PIP $_2$ (PDB id 3SPI), or with the anionic lipid pyrophosphatidic acid (PPA) bound (PDB id 3SPC). A structure without bound lipids has also been solved (PDB id 3JYC) and these different structures suggest an activation mechanism in which PIP $_2$ binding induces an upward translation and engagement of the C-terminal domain (CTD) with the transmembrane domain (TMD) [65, 58].

To explore the influence of these different initial conformations on these predictions as well as possible effects of the lipid bilayer, bound phospholipids are removed from the 3SPI and 3SPC Kir2.2 structures and both structures

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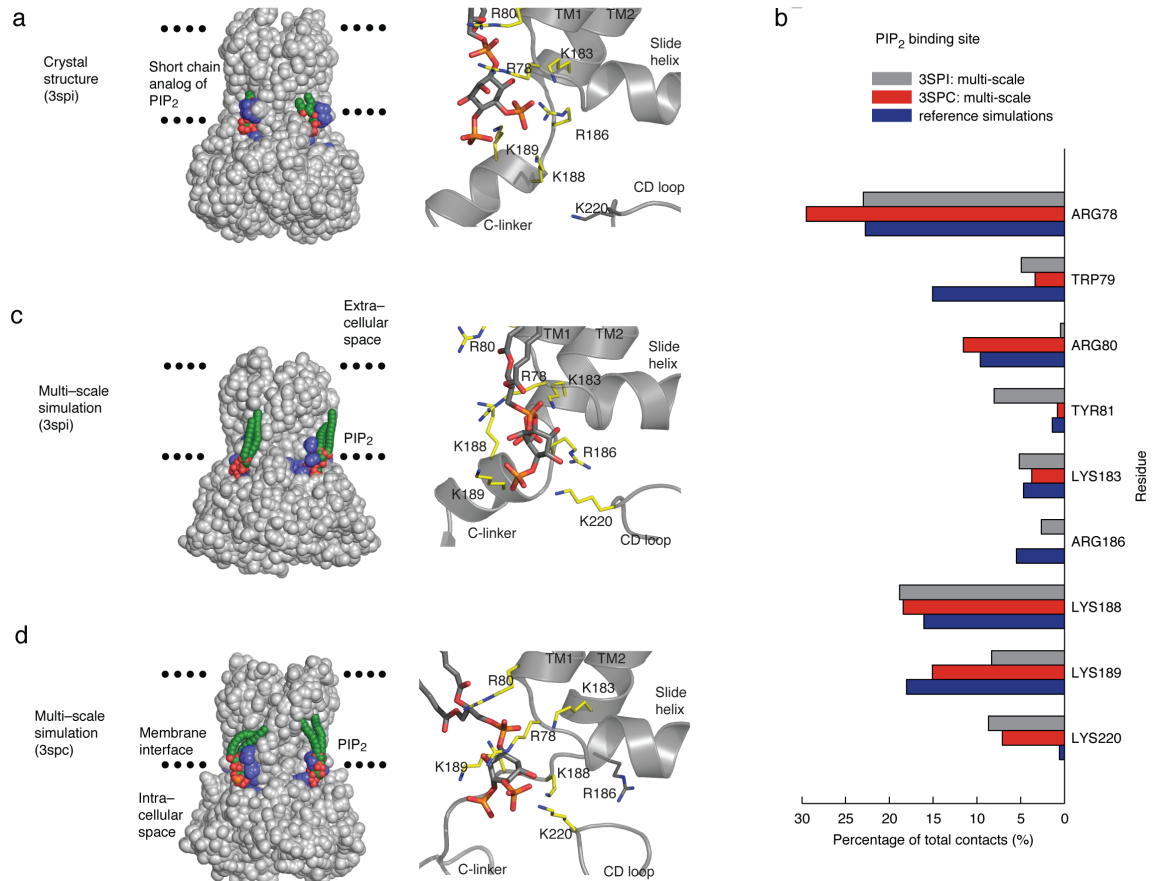


Figure 4.13: PIP₂ binding in Kir2.2 multiscale simulations. **(a)** Crystal structure with PIP₂ bound. For clarity, only two PIP₂ molecules are shown, with PIP₂ interacting residues in blue (left). Detailed view of the PIP₂ binding site (right) as found in the 3SPI structure. **(b)** Multiscale simulations used either 3SPI or 3SPC as starting structures and combined coarsegrained (CG; 24 × 0.5 μs) and atomistic (AT; 2 × 0.1 μs) simulations. Reference simulations were AT (2 × 0.1 μs) of the PIP₂ bound 3SPI crystal structure. Residues whose side chains make more than 5% of the total contacts (< 4 Å) with PIP₂ head groups are compared between the multiscale and reference simulations. **(c-d)** The binding site of PIP₂ predicted by **(c)** 3SPI or **(d)** 3SPC multiscale simulations.

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then used as input for multi-scale simulations of PIP₂ binding (Figure 4.13). To assess PIP₂ binding to a Kir2.2 lipid-unbound state, CG simulations are also performed using the 3JYC structure as input (Figure 4.3). In addition to comparing the predicted binding sites with the PIP₂-bound 3SPI crystal structure, atomistic simulations of this PIP₂-bound structure are performed. These reference simulations provide a more valid comparison with the multi-scale simulations, because it is then possible to directly compare simulations of both proteins embedded in a phospholipid bilayer at room temperature, rather than comparing a membrane-bound simulation with a static X-ray structure in the absence of a membrane.

4.4.1 The PIP₂ binding site in the PIP₂-bound crystal structure

Examination of the 3SPI crystal structure shows that residues which make contacts with the PIP₂ head group (Figure 4.13a) are mostly basic, and are either located on the N-terminal end of the first transmembrane helix TM1 (R78, W79, R80), or on the C-linker which connects the TMD with the CTD (K183, R186, K188, K189) [58]. In the reference simulation of this structure, all PIP₂-protein contacts were retained and accounted for nearly 90% of the total contacts made by side chains with the PIP₂ head groups (Figure 4.13b). Those residues which made less frequent contacts (5%) with the PIP₂ head groups (K183, R186) were located on the C-linker. The C-linker is helical in the PIP₂-bound 3SPI structure, but flexible in other Kir2.2 crystal structures, and may therefore account for the differences observed for K183 and R186 in these simulations. However, the reference simulations appear to confirm the stability of the 3SPI structure and of the PIP₂-protein interactions in this structure.

4.4.2 Agreement between simulations and the crystal structure

Importantly, the multi-scale simulations which used the protein coordinates from 3SPI as a starting structure predicted the PIP₂ head group to bind to the same cluster of basic residues as observed in the crystal structure (Figure 4.13c). This is in good agreement with both the reference simulations of this structure, with earlier predictions for other Kir channels [162, 286], and with most recent docking simulations [292]. Residues on TM1 (R78) and on the C-linker (K183, R186, K188, K189) were found to form the same number of contacts in the 3SPI multi-scale simulations as in the reference simulations (Figure 4.13b). Residue W79 on TM1 formed fewer contacts in the multi-scale simulations (5%) compared to the reference simulations (15%). The absence of any specific hydrogen bond interactions of W79 with PIP₂ in the crystal structure is likely to render the orientation of this side chain more flexible and might be the reason for less frequent contacts between W79 and PIP₂ in the multi-scale simulations compared to the reference simulations. Although W79 is suggested to facilitate PIP₂ binding by anchoring TM1 at the membrane interface [58], less frequent contacts argue against a direct interaction of W79 with PIP₂. The two adjacent residues (R80 and Y81) also exhibit differences between the multi-scale and reference simulations but this is most likely because of changes in TM2 which occur during the onset of channel gating. Nevertheless, these results demonstrate agreement between the PIP₂-bound 3SPI crystal structure, the 3SPI multi-scale simulations and the reference simulations.

4.4.3 Predicted binding site is independent from input structure

In addition to using 3SPI, the 3SPC protein coordinates were also used as an input for the multi-scale predictions. This structure was crystallized with the anionic phospholipid PPA instead of PIP₂ and more closely resembles the holo-state structure (3JYC) in which the CTD is detached and not fully engaged with the TMD [58]. Strikingly, when the ligand-free 3SPC structure was used as a starting point for the multi-scale simulations of PIP₂-binding, there was also good agreement between the multi-scale simulations and reference simulations (Figure 4.13d). Almost all contacts that were observed in the reference simulations were predicted by the 3SPC multi-scale simulations (Figure 4.13b) and were similar to those using 3SPI as a starting structure. The only major difference was the absence of any interaction between the head group of PIP₂ and R186 (Figure 4.13b). This is probably because in the 3SPC starting structure this C-linker is less well ordered and the R186 side chain points in the opposite direction when compared to the PIP₂-bound 3SPI structure. Longer simulations may therefore be needed to see if R186 changes its orientation to interact with the PIP₂ head group.

4.5 Discussion

The study of Kir2.2 gating mechanisms and PIP₂ binding has demonstrated the importance of the conformation of the C-linker in PIP₂-induced activation of the Kir2.2 channel. What is the relevance of this finding against the backdrop of the existing functional data and how compatible is this result with the recent

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experimental findings on PIP₂-Kir2 interactions [292, 140]?

4.5.1 Comment on C-linker helicity and opening of the HBC

The simulations on Kir2.2 presented here show that R186 affects the energetic barrier for K⁺ ion permeation and that a switch in the R186 side chain orientation, arising from PIP₂ binding, or a charge neutralisation, as in the R186A mutant, markedly decreases that barrier. This decrease arises primarily from a change in the effective electrostatic potential along the pore axis, rather than from an increase in pore radius.

What is the functional effect of neutralising the positively charged R186 on PIP₂-driven activation of Kir2 channels? Similarly, what is the effect of the R186A (and R186D) mutation on PIP₂ binding [292]? In liposomal ⁸⁶Rb⁺ uptake assays, in the absence of other proteins or intracellular pathways, R185Q primarily shifts the K_{1/2} of Kir2.1 activation, whereas the maximum flux remains unaffected by the mutation. Possibly the R186 charge neutralization does not largely affect channel conductance, for its contribution to the energy barrier at the HBC gate seems to be compensated by the PIP₂-induced reorientation of the C-linker and the R186 side chain. In other words, the structural changes following PIP₂-binding have the same effect as the R186 neutralization, as far as the electrostatic permeability is concerned. Because of the reorientation of R186, the contribution of the amino acid at position 186 to the energy barrier at the HBC is the same in the PIP₂-bound R186A channel as the PIP₂-bound R186 wild-type channel.

Kir2.1 channels are selectively activated by PIP₂, with weak or no activation by other PIPs [289, 290]. Unexpectedly, PIP₂-selectivity of Kir2.1 channel acti-

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vation is unrelated to the specificity of PIP binding interactions [292]. It was therefore proposed that differences in the coupling to channel opening, rather than differences in the residues controlling PIP binding, are determining the selectivity for PIP₂-induced activation of Kir2.1.

Specificity for activation by phosphoinositides across the Kir family is diverse [129]. Notably, in Kir3 channels, channel activation is not strongly selective for PIP₂ [290], and there is no selectivity at all in Kir6 channel [291], which are even activated by the complex anionic lipid long-chain CoA. Incidentally, these channel families have a glutamine at the position equivalent to R186 in Kir2.2 [291]. This suggests that the lack of charge of the residue equivalent to R186 in Kir3 and Kir6 channels may, among differences in structure and function [307], be responsible for the non-specific coupling of PIP binding to Kir3 and Kir6 channel activation, and thus for the non-specific sensitivity of Kir3 and Kir6 channels to PIP-induced activation. The binding site for PIP₂ in Kir3.2 is in close proximity to that found in Kir2.2 [251, 284] and thought to be near the predicted PIP₂ binding site in Kir6.2 [294]. It is therefore plausible that the charge on R186 and the change in orientation of the R186 side chain may govern the specific coupling of PIP₂ to Kir2, and other selectively PIP₂-activated Kir channels, and may contribute to the selectivity of these channels to PIP₂-induced activation. Consistent with this interpretation of the computational findings presented in Chapter 4 is the effect of mutating the residue corresponding to R186 in the highly specific channel Kir2.1 to the corresponding residue found in the least specific channel, Kir6.2. There, Rohacs *et al.* found that K185Q-Kir2.1 channels exhibit a specificity profile for PIP-induced channel activation that resembles the least specific channel Kir6.2 [291].

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D'Avanzo *et al.* suggest that sensitivity does not directly arise from the specificity of PIP binding [292]. They propose that the coordination of side chains in interactions with individual PIPs underlies PIP₂-specific sensitivity to activation. Here, an explicit model for PIP-induced Kir channel activation is provided, in that the orientation of the C-linker significantly affects the electrostatic energy profile of K⁺ ion permeation. The R186 side chain orientation is critically dependent on the C-linker, and the C-linker bears the primary responsibility for coupling of PIP₂ binding to Kir channel activation. In direct contrast to the model proposed by D'Avanzo *et al.* [292], the work described in this chapter suggests that the role of R186 is not only to bind PIP₂, but also to enable the transition to channel opening, which helps to explain the specificity of Kir2 sensitivity to PIP₂-induced channel activation, and may further our understanding of the specificity of PIP-induced Kir channel activation of other Kir channel family members.

4.5.2 Validation of a prediction method for lipid binding sites

The work described in this chapter also demonstrate that the multi-scale simulation approach can accurately predict the binding site for PIP₂ in Kir2.2 even when slightly different input structures are used. The principal advantage of the multi-scale approach compared to conventional docking methods is that both PIP₂ and the Kir2.2 channel are flexible. This can therefore provide insights into the molecular mechanisms underlying PIP₂ binding, such as conformational changes or an electrostatic contribution (Figure 4.2), which more static docking approaches lack. Interestingly, during the simulations the phosphates of the PIP₂ head group formed transient hydrogen bonds with K220 on the CD loop.

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This resulted in a decrease of the C_{α} - C_{α} distance between K220 and a highly-conserved aspartate on the slide helix (D76) by 2-4 Å. This motion appears to be due to the CD loop moving upwards towards the slide helix and C-linker during PIP₂ binding, similar to the gating mechanism proposed in several other studies [251, 252] and which may form part of a general activation mechanism for Kir channels by PIP₂ [286, 287]. The results also suggest that PIP₂ binding to the C-linker stabilizes its α -helical structure, and may account for the interaction of PIP₂ with R186 in the 3SPI crystal structure. Moreover, using the multi-scale approach, the effect of the lipid bilayer composition on PIP₂ binding can be studied. Studies on liposomes of controlled lipid composition highlighted an anionic secondary lipid requirement for PIP₂-induced Kir channel activation [140]. The CG simulations were therefore repeated, with half of the lipids on the inner leaflet replaced with the anionic phospholipid phosphatidylserine (PS). Notably, PIP₂ still binds to Kir2.2, while PS, accumulating on either sides of the slide helix, distributes unevenly around Kir2.2 (Figure 4.14). This suggests a secondary binding site for anionic lipids in Kir channels. Interestingly, this secondary anionic lipid binding site has recently been studied in greater detail in a docking-based prediction study of Kir2.1/lipid interactions, confirming the result obtained by these multi-scale simulations with PS [292].

4.6 Summary

In Kir2.2, small changes in the side chain orientation of a single arginine residue located at the PIP₂-binding site have a significant effect on the electrostatic barrier of K⁺ ion permeation. An open model of Kir2.2 was created without intro-

4. Kir channel gating and lipid interactions

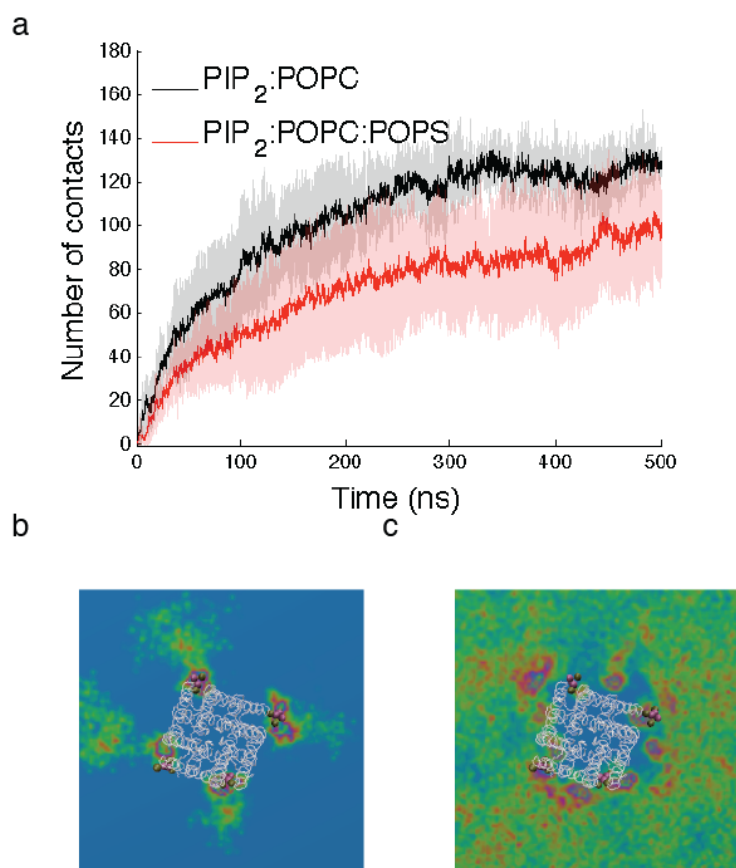


Figure 4.14: PIP₂-binding to Kir2.2 in the presence of PS. (a) Comparison between the average number of contacts between PIP₂ head groups and Kir2.2 (PDB id 3SPC) in coarse-grained (CG) simulations, for a neutral POPC bilayer (black), and for a bilayer in which half of the inner leaflet POPC molecules were replaced by the anionic lipid POPS (red). (b) Average distribution of PIP₂ (left) and POPS (right) around Kir2.2 (PDB id 3SPI) during a CG simulation, with half of the inner leaflet POPC molecules replaced by POPS. Color indicates low (blue) or high density (red) of normalized lipid distribution. Four PIP₂ molecules are bound in this CG simulation. VMD and Pymol were used for visualization.

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ducing activatory mutations, in which modelling the C-linker as a continuous helix clearly stabilised the HBC gate in an open conformation. Interestingly, the new model (17 Å after 0.1 μ s) opened to the same extent as the apparent open KirBac3.1 structure examined in the preceding chapter (17.2 Å).

The method to predict lipid binding sites to ion channels using a combination of coarse-grained and atomistic simulations now provides an important and powerful tool to help study protein-lipid interactions and will become increasingly important with the ever expanding number of high-resolution membrane protein crystal structures now available. The next chapter will employ this approach to probe PIP₂ binding sites of an ion channel that for which no crystal structure exists (as of September 2013).

Chapter 5

K2P channels

5.1 Introduction

Twin pore potassium (K2P) channels are lipid-regulated polymodal background ion channels that transduce a wide range of cellular signals into electrical activity. They therefore represent excellent candidates for the study of the structure and function of K2P channels in the context of a lipid membrane, and have been implicated in a wide range of diseases. The problem with the study of these channels is the limited availability of crystallographic structural information, which has constituted a considerable challenge for the development of reliable homology models and thus for the interpretation of functional data. In this chapter, a simple and fast 'ensemble' homology modelling method is developed. Following selection of the most suitable model, lipid/channel interactions of the PIP₂-sensitive K2P channel TREK-1 will be predicted and a model of the K2P channel TRESK will be presented. TREK-1 and TRESK channels are involved in pain pathways, general anesthesia, and migraine with aura and

better understanding of their function also of interest from a drug discovery viewpoint.

5.1.1 Previous modelling studies of K2P channels

The first homology models of TREK-1 were based on the structures of the bacterial KcsA channel [308], and on a composite template comprising the X-ray structures of KcsA and a phosphate acetyl transferase [98]. After these initial studies, Piechotta *et al.* built a TREK-1 homology model based on the structure of the prokaryotic KvAP channel, which they compared with these earlier models and with models based on more remotely related K⁺ channels [34]. Scanning mutagenesis of the TREK-1 channel pore identified a quaternary ammonium (QA) ion-binding site in TREK-1 [34] and by comparing this binding site with *in-silico* predictions, Piechotta *et al.* ranked all available TREK-1 models. The TREK-1 model based on the KvAP structure presented the best fit to their functional scanning mutagenesis data [34].

5.1.2 Recent X-ray structures of K2P channels

More recently, X-ray crystal structures of two related human K2P channels, TRAAK and TWIK-1, were determined [86, 90]. Homology between K2P channels is moderate, as low as 20%, but TREK-1 shares an exceptionally high sequence identity of 45% with TRAAK, and 28% with TWIK-1 [80, 66]. Therefore, by taking advantage of these new structures, an improved homology model of TREK-1 will be developed. Models of the human TREK-1 channel based on KvAP, TRAAK, and TWIK-1 are created, and QA ions docked into each homology model. The results from these dockings are then compared to

Piechotta *et al.*'s functional scanning mutagenesis data and to a new TREK-2 X-ray crystal structure determined by Carpenter and co-workers (PDB id 4BW5, to be published). The best-fit model will be selected and used for the study of lipid/TREK-1 interactions. These new homology models may be used to as tools for binding site predictions of drugs or synthetic lipids. In combination with experimental assays, the effect of mutations on the inhibitory or activatory effect of such drugs or lipids may be studied experimentally, or with MD or docking simulations, using models created in this chapter. New structures of K2P channels may serve as templates for even better homology models, which may be created following the procedure developed and presented in this chapter.

5.2 Methods

5.2.1 Modelling

To form TREK-1 homology models based on TRAAK, an alignment was built between TREK-1, TWIK-1 (PDB id 3UKM) and TRAAK (PDB id 3UM7), using MUSCLE [309], which was then manually refined in Jalview [310]. Manual refinement considered conservation of hinge glycines, prolines in TM4, and helix-delimiting glutamate residues. TREK-1 models were generated as a multimer, with protomer 1 of the model build on protomer 1 of the template structure, and vice versa for protomer 2. The C-terminal end of the extracellular loop connecting the selectivity filter and the cavity-lining TM4 of pore domain 2 corresponding to residues Y281 - Y285 in TREK-1 was modelled on TWIK-1.

All other parts of the model are based on TRAAK.

The final TREK-1 model based on TRAAK consists of residues 57 - 318. The accuracy of loop-forming residues 137 - 141, and 216 - 217 in chain A, and residues 135 - 141, and 207 - 220 in chain B is uncertain, as the structure of the corresponding template residues has not been resolved and the quality of the model in these domains could thus not be assessed (Figure 5.1). These residues form the loop between the extracellular cap helix and the pore helix 1, and the connection between the intracellular ends of the inner TM2 and the TM3. Loop modelling was not used for refinement, as these domains are unlikely to affect the binding site of quaternary ammonium ions (QA). Symmetry restraints were imposed during the modelling procedure between the C α -atoms of chain A and chain B. The other TREK-1 models were built analogously, using either TWIK-1 or KvAP as template.

5.2.2 *In silico* small molecule docking

QA ions of varying sizes were docked into the cavities of the TRAAK-based, KvAP-based, and TWIK-1-based models of TREK-1, and into the TRAAK structure. Alkyl chain lengths varied between 2 - 8 Å. Dockings were performed using Autodock 4.2 [241]. Ligand and receptor molecules were configured using Marvin 5.5.1 (ChemAxon) and AutoDockTools 1.5.4 [312]. PBD2PQR [256] was used to add missing atoms, and AutoDockTools 1.5.4 [313, 167, 314] to add polar hydrogens, atom types, and partial charges. QA ions were docked into each model with and without restraints applied to the nitrogen atom, employing 20 runs per docking and 2,500,000 evaluations of the Lamarckian-Genetic-Algorithm [241]. Without restraints, QA ions were docked in a small grid box

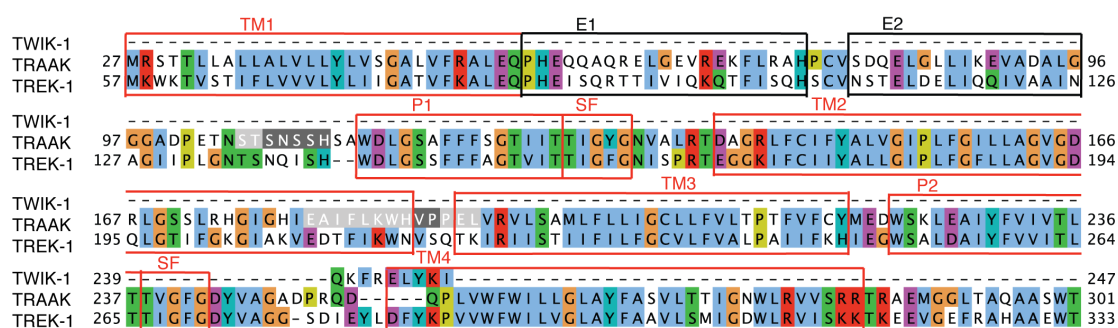


Figure 5.1: Alignment of hTWIK-1 (Uniprot: O00180), hTRAAK (Uniprot: Q9NYG8), hTREK-1 (Uniprot: O95069) used to generate the TRAAK-based TREK-1 models. TWIK-1 residues which are not used as input for MODELLER are hidden from the alignment. The ClustalX [311] default colour coding is used. TREK-1 residues following TM4 (residues 319 - 333) presumably form an amphiphatic helix but could not be modelled as the electron density data is not available. Residues on grey or dark grey background were not observed in the TRAAK structure. Residues on grey background were not resolved in one of the two protomers, while residues on dark grey background were not resolved in either of the two protomers of the TRAAK structure (PDB id: 3UM7). Red boxes mark transmembrane domains, black boxes the extracellular cap [86]

with a side length of 11.25 Å, corresponding to 30 x 30 x 30 grid points. The grid was chosen large enough to accommodate the entire cavity including the surrounding transmembrane helices. With restraints, the nitrogen atom of the QA ion was aligned and restrained to the binding site of TButA found in the 2JK5-KcsA [31]. Residues were recorded if their side chain came closer than 4.0 Å to any atom of the QA ion.

5.2.3 PIP₂ binding simulations

In order to examine PIP₂ binding in coarse-grained simulations, PIP₂ molecules were embedded in the inner leaflet of a phosphatidylcholine (PC) bilayer, distributed at random positions in distances between 10-15 Å from the centre of TREK-1. In total, 5 % of the total number of PC lipids was replaced by PIP₂ molecules. In CG simulations with phosphatidylserine (PS), 25 % or 65 % of the total inner leaflet PC lipids were randomly replaced by PS. Unlike PIP₂, placement of PS was not confined to a specific distance from TREK-1. Run-time parameters were set as in the preceding two chapters. The simulation time was 0.5 μ s for each configuration. 3 x 10 simulations were run for the TRAAK/Fab-based TREK-1 model configuration and 3 x 5 simulations for the TRAAK-based TREK-1 model configuration, each set of the three sets using different lipid compositions (95 % PC : 5 % PIP₂, 70 % PC : 25 % PS : 5 % PIP₂, 30 % PC : 65 % PS : 5 % PIP₂). Residues E320 - H 328 were modelled as helix *ab initio*. These residues include a patch of five basic residues that abrogate PIP₂ sensitivity upon mutation or truncation [99]. Deletion of the C-terminus after R326 was shown to retain PIP₂ sensitivity [99].

5.3 TRAAK- and TWIK-1-based K2P models

In this section, homology models of TREK-1 are developed and compared to the functional scanning mutagenesis data of Piechotta *et al.* [34]

5.3.1 TPenA binds to the pore of 'open' TREK-1 models

The two new homology models of TREK-1 are based on the closely related TRAAK structure (PDB id 3UM7) and on the TWIK-1 (PDB id 3UKM) structure. In these new models, in agreement with the functional scanning mutagenesis and with the earlier KvAP-based model (Figure 5.2a), *in-silico* docking of the QA ion tetrapentylammonium (TPenA) predicted a binding site deep inside the pore, just below the selectivity filter (Figure 5.2b). In the new TREK-1 models, this prediction is also consistent with the binding sites of QA ions in KcsA structures (PDB ids 2JK5 and 1J95 [31]) and with the binding site of the channel blocker A1899 in TASK-1 [315].

The pore size at the helix bundle crossing in the new model is clearly open (pore radius > 6 Å). Intracellular acidification and membrane stress change TREK-1 open probability, while leaving the inhibitory potency (IC_{50}) of TPenA block unaffected [34]. Together, these results suggest that the helix bundle crossing is 'open', even if the channel is in a closed conformation [94, 95, 92], supporting the hypothesis that this channel gates at or near the selectivity filter, rather than at the helix bundle crossing [316, 317, 34].

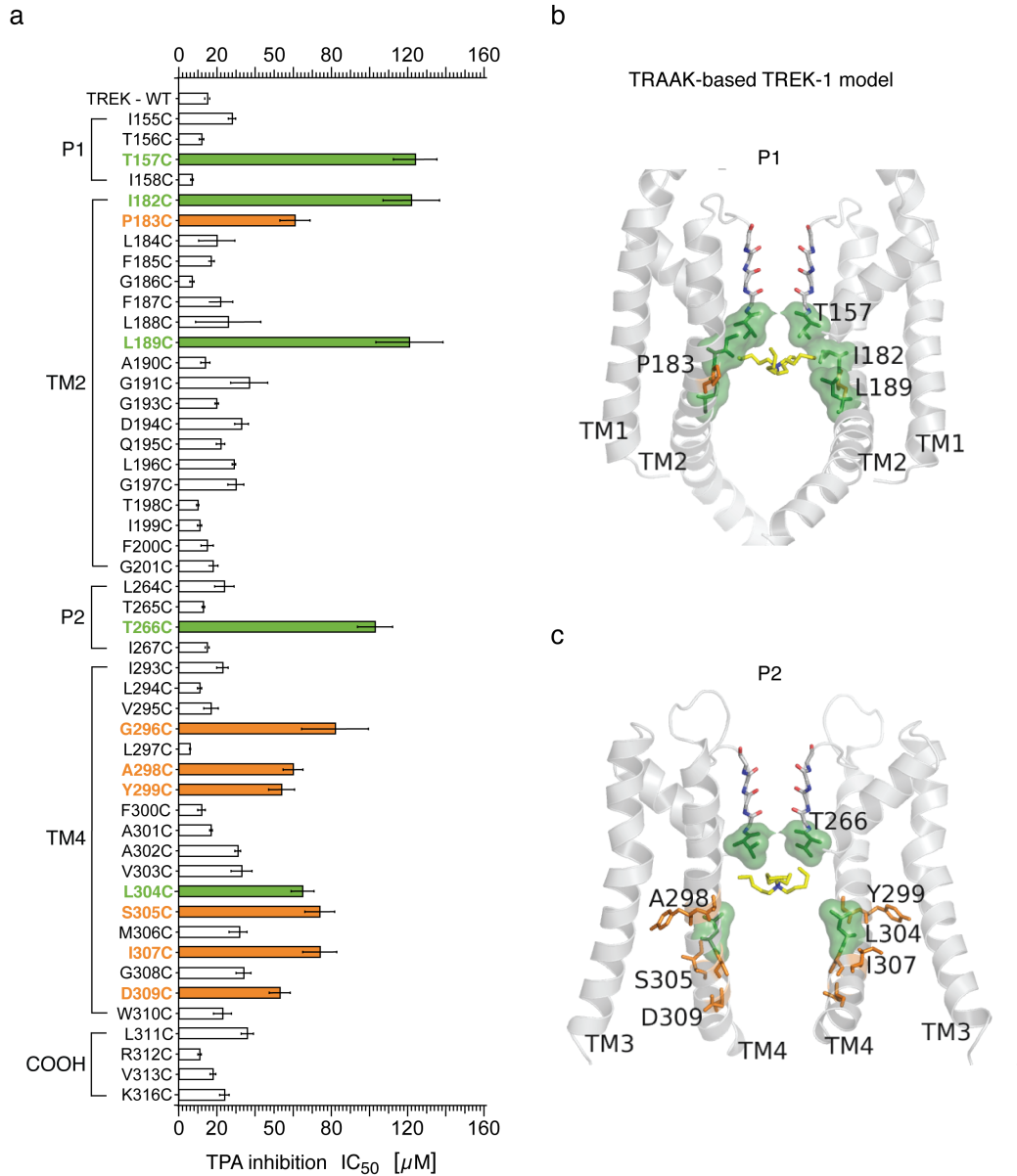


Figure 5.2: (a) mapping of residues affecting channel inhibition by quaternary ammonium blockers on a TRAAK-based model of TREK-1. (b) mutation of residues in shown in green affects channel inhibition by mutation of the blocker binding site. Residues in orange prevent blocker inhibition through an allosteric mechanism. (a) Taken and adopted with permission from [34].

5.3.2 Ensemble docking allows scoring of TREK-1 models

Piechotta *et al.* built TREK-1 models based on structures of distantly related bacterial channels (KvAP, KcsA, MthK, Kv1, NaK, and MlotiK). Scanning mutagenesis of the TREK-1 channel pore for residues affecting TPenA inhibition identified a TPenA binding site near the selectivity filter, in agreement with the binding site found in the KcsA X-ray structure ([31, 34]).

To evaluate these TREK-1 models, Piechotta *et al.* compared the TPenA binding site (obtained from functional mutagenesis) with TPenA binding sites derived from *in-silico* docking of TPenA into the TREK-1 homology models. This comparison identified (for each model) ‘hits’, defined as residues that affect TPenA inhibition upon mutation and that are $< 4 \text{ \AA}$ away from docked TPenA. Furthermore, false positives could be identified, defined as residues that were predicted by *in-silico* dockings to form part of the binding site, but that did not affect TPenA inhibition upon mutation. Finally, Piechotta *et al.* scored their TREK-1 homology models according to the number of false positives.

Here, the same approach was followed, and new TREK-1 models were built based on the homologous templates of the new X-ray crystal structures of TWIK-1 and TRAAK. However, the *in-silico* dockings of TPenA into these new TREK-1 models did not lead to reproducible results. Presumably, the TRAAK-based and TWIK-1-based homology models were structurally too similar, which is perhaps not surprising given the homology of these templates [66]. Therefore, in this thesis, an ensemble of TREK-1 models ($n > 100$) was generated for each template using MODELLER [225]. TPenA was then docked into each ensemble of TREK-1 models using an automated modelling/docking

routine.

This approach has two advantages: first, taking the ensemble average is likely to return a more reliable measurement of ‘hits’ and false positives than docking into one single, arbitrarily chosen structure. Secondly, docking software such as AUTODOCK [241] does not allow full flexibility of the protein structure. However, as the MODELLER-generated ensembles include structurally different models, the TPenA molecule could effectively be docked into pseudo-flexible TREK-1 homology models.

In summary, this new ensemble docking method imitates previously unavailable full flexibility of the binding site, and returns a quantifiable measure of the propensity of each individual residue to interact with TPenA, allowing clear distinction between ‘hits’ and false positives across closely homologous templates. Thus, the new TREK-1 homology models could be scored.

5.3.3 Scoring of new TREK-1 models

The TPenA binding site in the new TREK-1 homology models based on KvAP, TWIK-1, and TRAAK, was calculated using the new ensemble docking method. ‘Hits’ are shown in green, false positive interactions with TPenA in red (Figure 5.3). For each template structure, dockings were performed twice: with the central nitrogen (N) of TPenA restrained to the position of a corresponding QA ion in the 2JK5-KcsA crystal structure [31], and with a fully flexible TPenA ligand.

In all models, residues T157 and Y266, which form part of the highly conserved K⁺ selectivity filter (TXGYG) signature sequence (Figure 5.4), interacted directly with the N atom of TPenA (> 95 % of all dockings), which is consistent

with the binding site of QA ions in the KcsA crystal structures. Importantly, this direct interaction was observed as often in flexible dockings as in dockings where the N atom was restrained.

The hydrophobic alkyl tails of TPenA also made direct interactions with the L189 side chain on TM2. This interaction was more pronounced in flexible ligand dockings (> 60 % for KvAP- and TRAAK-based models) and least in models based on TWIK-1 (45 %). Although it is not obvious that residues which affect ligand inhibition upon mutation are part of the binding site [295], the T157C-L189C double mutant produced a 500-fold reduction in TPenA sensitivity and affected TPenA inhibition in an additive manner [34]. Therefore it is likely that residues T157 and L189 are both part of the TPenA binding site [318]. Interestingly, L304, the equivalent of L189 on TM4 (Figure 5.4), displayed frequent interactions with TPenA only in KvAP-based models (70 % - 90 %), whereas it showed none or few (at most 20 % in flexible dockings, < 1 % with the N-atom restrained) interactions in TRAAK- and TWIK-1-based models (Figure 5.3).

All three templates (KvAP, TRAAK, and TWIK-1) generated TREK-1 models which interacted with TPenA through residue I182 on TM2 (> 90 % in flexible dockings, 50 % - 90 % with the N atom restrained). I182C reduced TPenA sensitivity *in vitro* ca. 100-fold (Figure 5.2a), and this residue is therefore also likely to form part of the TPenA binding site. The equivalent residue of I182 on TM2, L297 on TM4 (Figure 5.4), which did not reduce TPenA sensitivity *in vitro* (Figure 5.2), made frequent false positive interactions with TPenA in KvAP- and TWIK-1-based models (ca. 80 %). Surprisingly, these false positive interactions with L297 were reduced two-fold in the TRAAK-based models (45 % in flexible

5. K2P channels

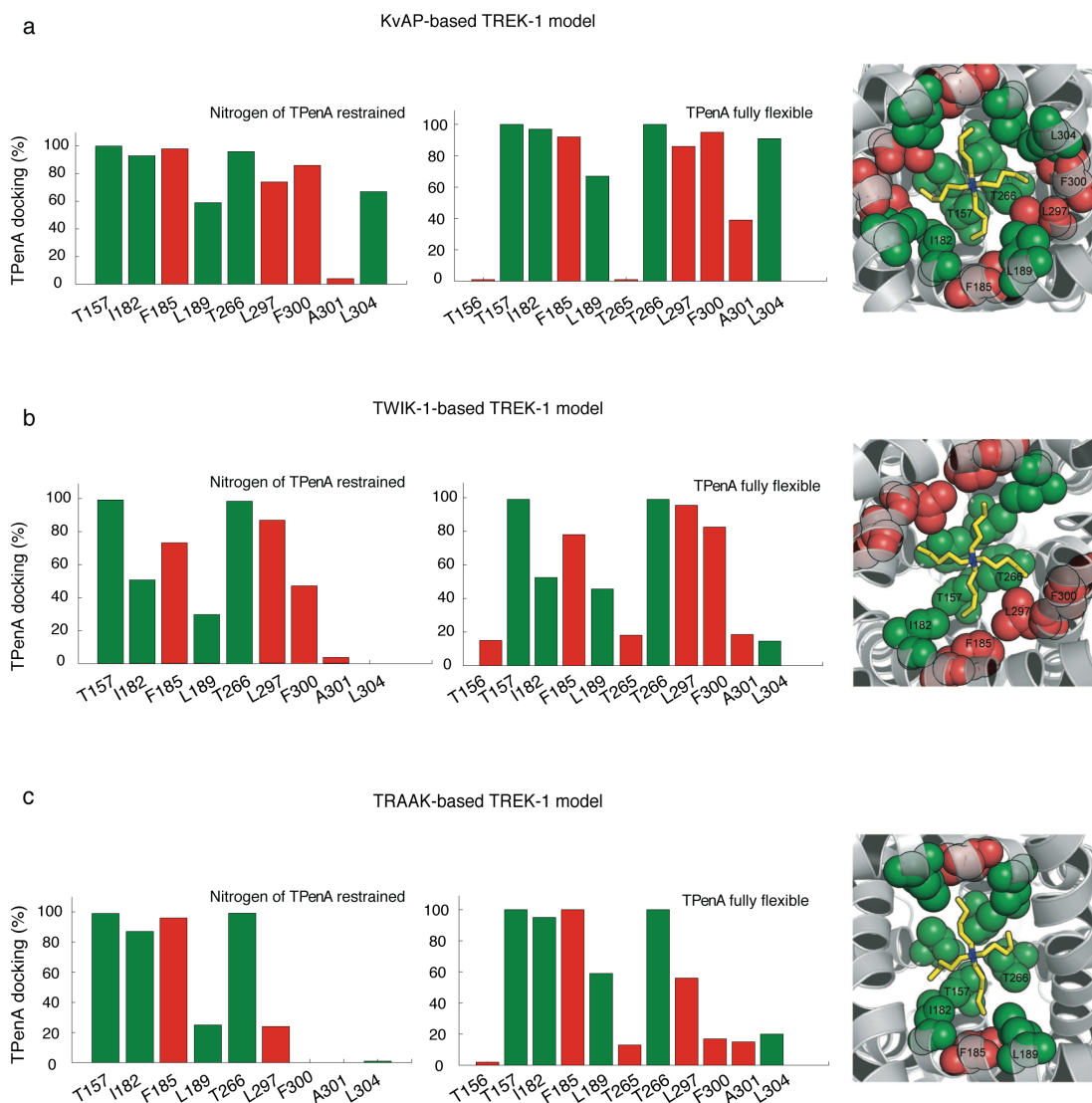


Figure 5.3: Ensemble dockings of TPenA into TREK-1 models. (a) KvAP-based, (b) TWIK1-based, and (c) TRAAK-based TREK-1 models. 'Hits' are shown in green, false positive interactions in red. Dockings were performed with the central nitrogen of TPenA restrained to the location found in the crystal structure by Zhou et al. [31] (Nitrogen of TPenA restrained), and without restraints (TPenA fully flexible). TRAAK-based models exhibit fewer false positives compared to KvAP-based or TWIK-1-based models.

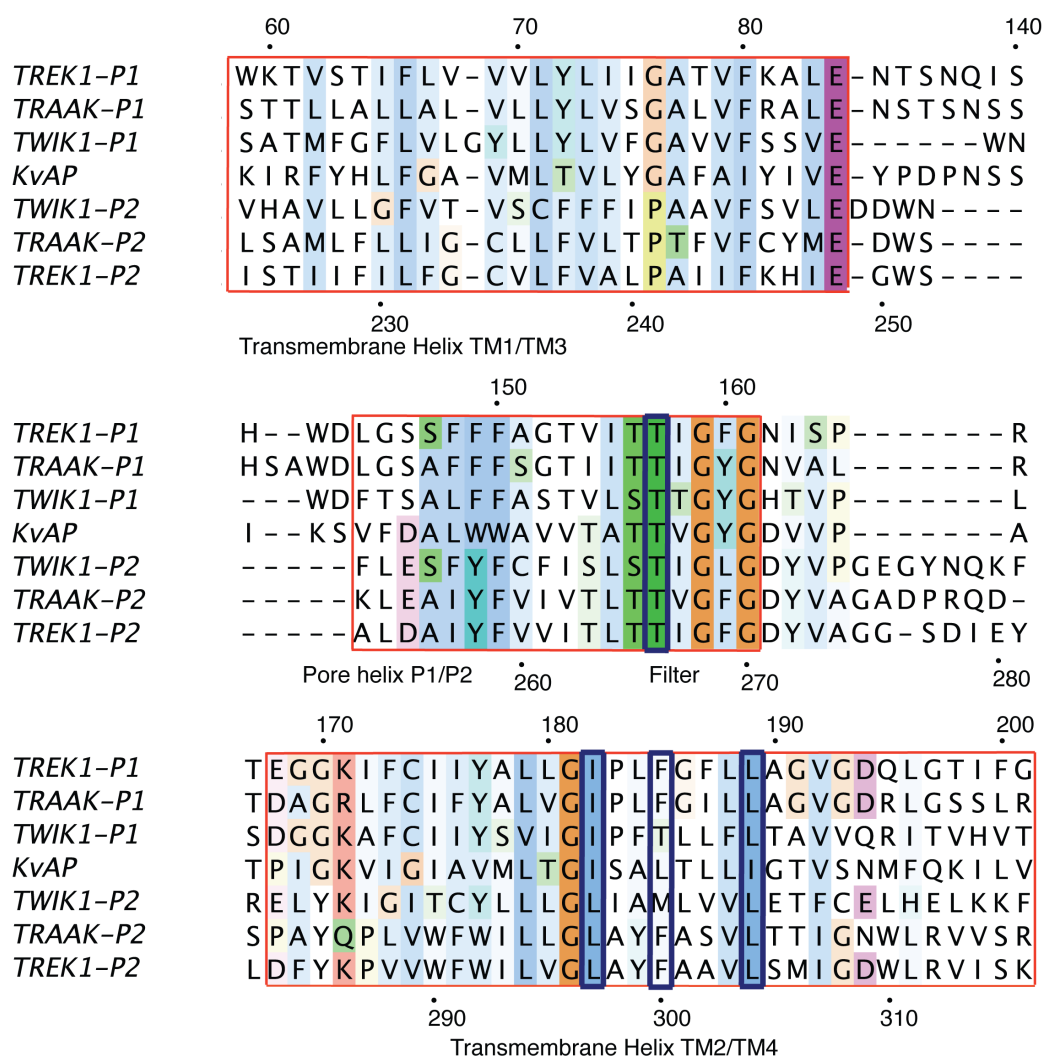


Figure 5.4: Alignment of TREK-1, TRAAK, TWIK-1, and KvAP sequences. Transmembrane and pore helices are marked by red boxes. Blue boxes highlight residues which are predicted to interact with TPenA *in silico*. Figure rendered with Jalview [310].

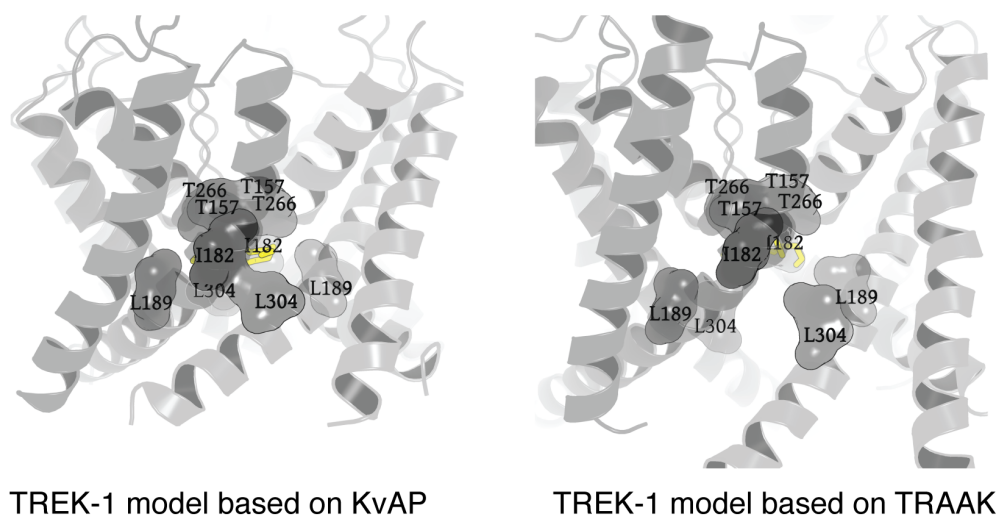


Figure 5.5: Model of TREK-1 based on (a) TRAAK and (b) KvAP, with TPenA docked in the pore (yellow). The backbone of TM4 of the TRAAK-based model is displaced compared to the KvAP-based model. This asymmetry in the arrangement of pore-lining helices of the TRAAK-based model results fewer false positives in *in silico* dockings of TPenA.

dockings, 20 % with the N atom restrained). This indicates that TM4, crucial for the transduction of intracellular signals to the gate at the selectivity filter [94, 95, 317], likely occupies different conformations in the mechano-sensitive TREK-1 and TRAAK channels compared to the less homologous TWIK-1 and KvAP channels [80, 91, 90] (Figure 5.5).

In all models of TREK-1, F185 in TM2 made very frequent (70 % - 90 %) contacts with TPenA (Figure 5.3), although the F185C mutant did not reduce TPenA sensitivity *in vitro*. However, while 'hits' (I182, L189) in TM2 are conserved among all templates, conservation of F185 is at most moderate (Figure 5.4). Binding of TPenA may therefore be insensitive, *in vitro*, to the conservative F185C mutation. Interestingly, F300 on TM4, the residue equivalent to F185 on

TM2, only contacted TPenA in KvAP- and TWIK-1-, but not in TRAAK-based models. Furthermore, F300 did not reduce the sensitivity to TPenA inhibition upon mutation and therefore forms unlikely part of the binding site. Together, this demonstrates again the sensitivity of the predicted TPenA binding site to the TM4 conformation, which will be discussed in greater detail later (Figure 5.5).

Together, these dockings show that the TRAAK-based model of TREK-1 exhibits the least number of false positives. If F185 is excluded from the analysis, then the TRAAK-based model is the only model that exhibits no false positive interactions. Several residues affected the TPenA sensitivity in Piechotta's functional mutagenesis screen, and were not predicted to form part of the binding site in any of the models, e.g D309. Assuming that these residues alter TPenA binding through an allosteric or electrostatic mechanism (the D309C mutation resulted in a 20 - 25 kcal / mol decrease of the electrostatic pore profile at the helix bundle), the TRAAK-based models correctly identified four (T157, T266, I182 and L189) out of five possible (T157, T266, I182, L189 and L304) residues of the TPenA binding site. The role of L304 on TM4 in TRAAK-based models remains unclear.

5.3.4 *In-silico* predictions are sensitive to the orientation of TM4

The arrangement of false positives is four-fold symmetric in KvAP-based models, but only two-fold symmetric in TRAAK-based models (Figure 5.3). Consequently, TRAAK-based models had fewer false positives, and were in better agreement with the functional scanning mutagenesis data. It is tempting to propose that the origin of this break in symmetry is a tilt mid-way in TM4

around the absolutely conserved G296, which splays apart the C-terminal half of the pore-lining TMs in the TRAAK-based TREK-1 model (Figure 5.5). This movement appears to enable looser packing between TM2 and TM4 in the TREK-1 model, similar to the fenestration observed in TRAAK, and may allow an alternative access pathway for TPenA block. However, biochemical and electrophysiological experiments, showed that TPenA access to the binding site is state-independent and limited to permeation through the helix bundle, suggesting that pore access by the blocker occurs via the helix bundle rather than the fenestrations and that the helix bundle of TREK-1 is open in both the 'open' and closed conformations [316].

5.3.5 Size-dependence of QA block in TREK-1 models

To further verify the best-fit model, different sized QA ions were docked into the cavity below the selectivity filter of the TRAAK-based model (Figure 5.6a). The observed modes of binding were in good agreement with analogous docking simulations performed by Piechotta et al. [34]. In unrestrained dockings to the TRAAK-based model, consistent with the reported insensitivity of TREK-1 to small QA ions [66], TEthA did not bind to the center of the pore. The TEthA nitrogen atom was not coordinated by the cavity-lining threonines of the selectivity filter. Occasionally, TEthA ions escaped into the fenestrations between TM2 and TM4. By contrast, with increasing QA size (TPenA, THexA), the QA ion centered in the cavity, in a position similar to the binding site obtained from the functional mutagenesis scan. A further increase of the QA size beyond THexA resulted in steric clashes of the alkyl tails with the cavity-lining helices, in agreement with the experimentally observed lower affinity for these

blockers in TREK-1 [34].

To further investigate the size-dependence of QA block, the average free energy of binding for each QA compound was quantified for KvAP-, TRAAK- and TWIK-1-based models (Figure 5.6b). As expected, THexA displayed the lowest estimated free energy of binding in all three models. Interestingly, unlike in TRAAK-based models, docking into TWIK-1-based models resulted on average, in a lower predicted free energy of binding. This is likely a consequence of the large number of false positive interactions with TREK-1 in TWIK-1-based models (Figure 5.3) [318].

5.3.6 Building a TRESK model based on TRAAK

The human TRESK channel is involved in migraine with aura and exhibits a unique topology [67, 82]. Missense mutations of residues A34V and C110R lead to a reduction in TRESK channel activity [85]. A model of the human TRESK channel was developed, based on a) the TRAAK structure used in the previous section, and b) the TRAAK-Fab antibody complex determined more recently [91]. Both models were moderately similar to an earlier published KvAP-based model (4 Å) [85]. The most similar channel to TRESK is TREK-2 [67]. Thus, the high-resolution TRAAK structure (2.75 Å) may represent a good starting point for the development of future TRESK homology models.

The TRESK homology models based on KvAP, TRAAK, and the TRAAK-Fab complex are shown in Figure 5.7. Interestingly, residues A34 and C110 pack more closely in the TRAAK-based model than in the TRAAK/Fab-based model. Both residues are located next to the selectivity filter, which has been suggested to control K2P channel gating [316, 34, 95]. However, no functional

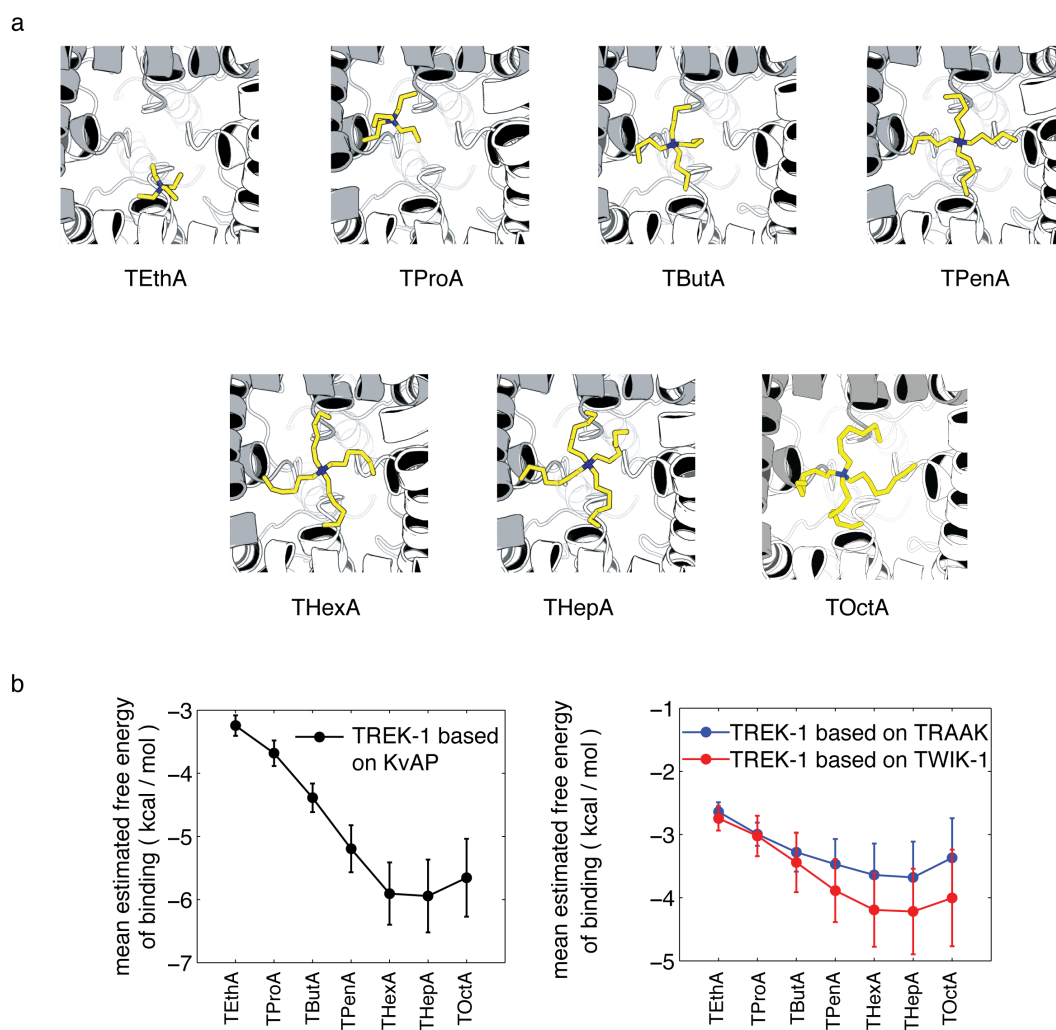


Figure 5.6: Ensemble docking of quaternary ammonium (QA) ions in TREK-1 models. (a) Small QA ions can not bind to the centre of the cavity. Large QA ions (THepA, TOctA) collide with cavity-lining helices. (b) The calculated free energy of binding is lowest for THexA. Binding of QA ions with long alkyl tails (THepA-TOctA) suffers from steric clashes with cavity-lining helices

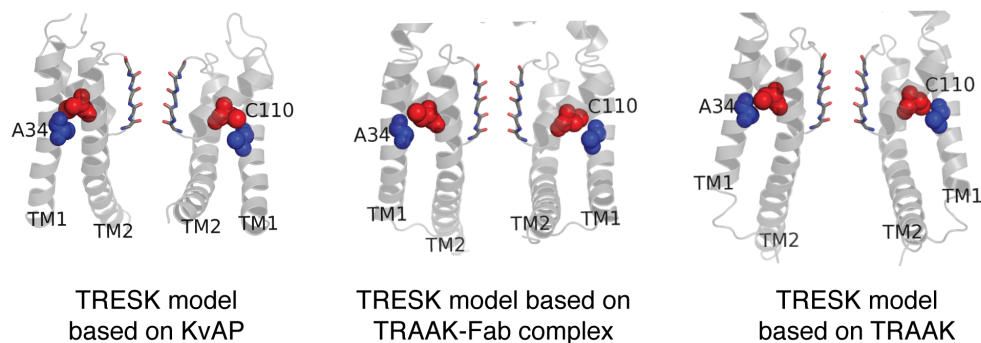


Figure 5.7: TRESK models based on TRAAK and KvAP structures. Disease mutants A34V, C110R are coloured blue and red, respectively, and are located close to the selectivity filter, which has been implicated in the control of K2P channel gating.

mutagenesis data was available for TRESK. Future models will improve with the availability of more closely homologous template structures. However, the intracellular domain of TRESK, for which no relative exists in the K2P channel family, constitutes a considerable challenge in modelling the structure and function of TRESK.

5.4 PIP_2 /TREK-1 interactions

In the preceding section, a homology model of TREK-1 was developed that agreed well with functional mutagenesis data. Here, this model will be used, along with a newer TREK-1 model based on the TRAAK/Fab-complex structure, to predict TREK-1/ PIP_2 interactions, using the simulation-based method validated in Chapter 4. Highly charged anionic lipids, such as PIP_2 , up- and down-regulate TREK-1 [96] in a complex manner [99, 146, 93]. However, only relatively indirect pharmacological approaches, such as Gq-coupled receptor

stimulation, application of inhibitors of phosphoinositide kinases or of phospholipase C, activation of PIP₂-depletion systems, such as a voltage-sensing phosphoinositide phosphatase from *Ciona intestinalis* [319] or rapamycin-inducible recruitment of a phosphoinositide 5 phosphatase enzyme to the membrane, can up- or downregulate endogenous PIP₂ concentrations. Likewise, exogenous application of PIP₂ to excised patches suffers from an unknown degree of incorporation into the membrane, which may lead to artificially high concentrations [108], and from unknown effects of the acyl chain length of exogenously applied PIP₂. PIP₂-regulation of the related TASK-1/TASK-3 channel is also subject of considerable speculation [12, 108, 320]. The following simulations will directly approach the question of the PIP₂ binding site in TREK-1.

5.4.1 Phosphatidylserine attenuates PIP₂ binding

By performing two sets of coarse-grained PIP₂-binding simulations, the robustness of PIP₂-binding predictions on TREK-1 was evaluated (Figure 5.8). For one set of simulations, the previously determined best-fit TRAAK-based model (PDB id: 3UM7) was used (Figure 5.8a), while for the other set of simulations a TREK-1 model based on the newer TRAAK/Fab-complex (PDB id: 4I9W) was employed (Figure 5.8b). In both sets of simulations, PIP₂ clearly binds to TREK-1. The addition of the anionic phospholipid phosphatidylserine (PS) to the inner leaflet of the lipid membrane attenuates PIP₂ binding, suggesting an electrostatic contribution to PIP₂ binding. TREK-1 activation is not limited to PIP₂, and includes sensitivity to PS. It is therefore not surprising that the number of contacts between TREK-1 side chains and PIP₂ head groups was decreased with increasing PS concentration in the inner leaflet. However, it is notable that,

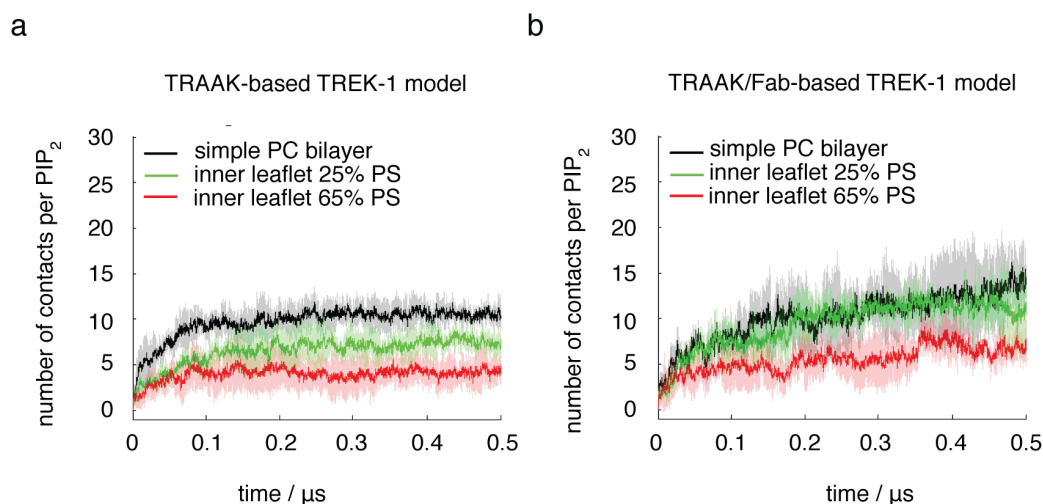


Figure 5.8: PIP₂ binds to TREK-1 homology models. **(a)** PIP₂ binding to TRAAK-based (PDB id: 3UM7) homology model. **(b)** PIP₂ binding to TRAAK/Fab complex-based (PDB id: 4I9W) homology model of TREK-1. Addition of the anionic phospholipid phosphatidylserine (PS) to the inner leaflet attenuates binding of PIP₂. A cut-off of 6 Å was used to count contacts between TREK-1 side chains and PIP₂ head groups.

upon addition of PS, the number of contacts between PIP₂ and TREK-1 was still ca. 10 times higher (5 - 10 lipid-protein contacts / PIP₂ molecule) than the number of contacts between PS and TREK-1 (0.5 - 1.5 lipid-protein contacts / PS molecule). On average, less than one protein contact per PS molecule indicates that not all PS molecules are bound to TREK-1, whereas 10 contacts per PIP₂ molecule or more suggests that all PIP₂ molecules bound to TREK-1.

5.4.2 Two basic clusters contribute to PIP₂-interactions

Examination of PIP₂-interacting residues reveals two basic clusters located at the C-terminal end of TM4 and the TM2-TM3 loop (Figure 5.9a). Mutation of

a patch of five basic residues on the C-terminal end of TM4 (R312, K316, K317, K319, and R326) abrogates PIP₂ sensitivity [99]. From this patch, which was not resolved in the TRAAK template, and which was modelled *ab initio* as a helix, R326 accounted for 3 - 5 % (Figure 5.9b) of the total number of PIP₂/TREK-1 interactions (twice as much than the average plus one standard deviation of the distribution). PIP₂/TREK-1 contacts with the TM2-TM3 linker (K202, K206, K213, K220) have not been identified in experiments so far. PIP₂ also made frequent contacts with the N-terminal KWK motif on TM1 in coarse-grain simulations, which is, in the model, located close to the C-terminal part of TM4. However, these residues (K58, W59, K60) are not conserved in the K2P family, although basic residues are quite common at the N-terminal end of TM1.

5.4.3 Comparison to lipid binding site in TREK-2 structure

Towards the end of the work performed for this thesis, Carpenter and co-workers determined a new crystal structure of TREK-2 (PDB id 4BW5). This structure was obtained in two conformations, a lipid-bound conformation, in which the C-terminal part of TM2 is tilted at the conserved hinge residue G191 which resulted in a ca. 5 Å upward shift of the distal end of TM2 towards the lipid bilayer, the 'up-state', and in an alternative conformation, the 'down-state' (Figure 5.10a). Interestingly, an alignment of PIP₂ to the co-crystallized lipid shows that the head group of PIP₂ could well interact with the basic residues (K213, R126) on the TM2-TM3-linker that were predicted in PIP₂/TREK-1 simulations (Figure 5.10b).

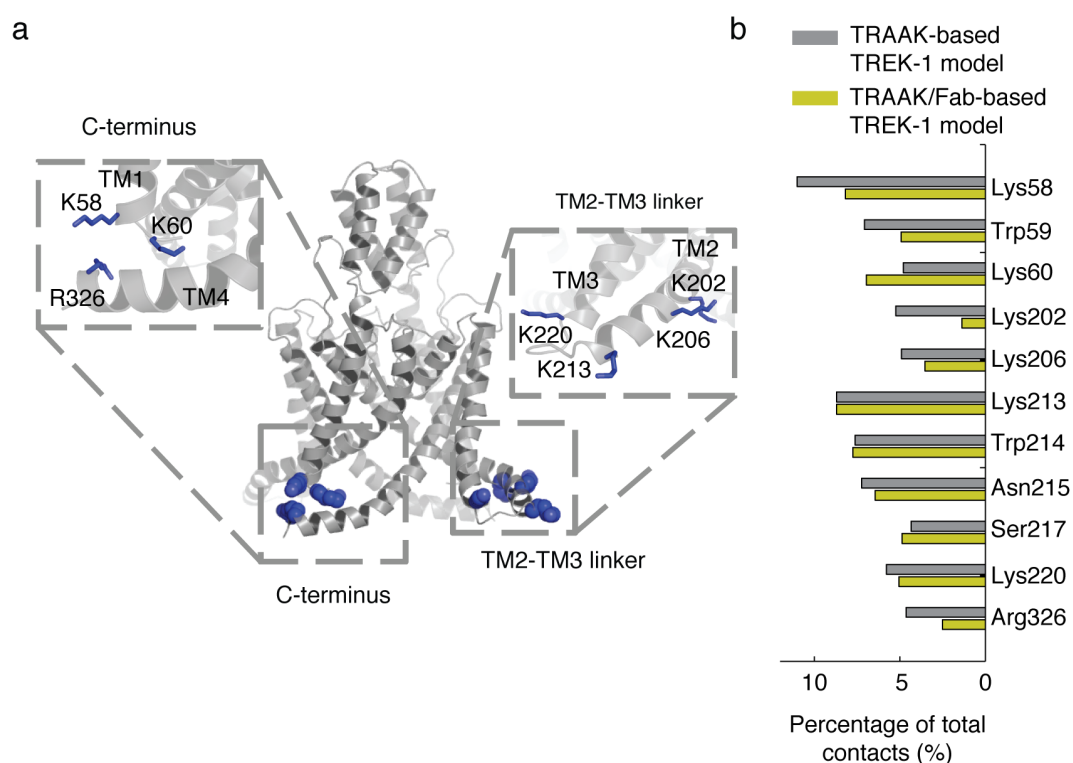


Figure 5.9: Predicted PIP₂-TREK-1 interactions. Two clusters of basic residues contribute to PIP₂/TREK-1 interactions: **(a)** The C-terminal end of TM4, and the TM2-TM3 linker. **(b)** All PIP₂-interacting residues predicted from coarse-grain simulations ($5 \times 0.5 \mu\text{s}$ for TRAAK-based TREK-1 model, $10 \times 0.5 \mu\text{s}$ for TRAAK/Fab-based TREK-1 model) responsible for $> 2\%$ of the total contacts. Interactions between TREK-1 side chains and PIP₂ head groups were counted during the last $0.25 \mu\text{s}$ of each trajectory using a $6 \text{ \AA}$ cut-off.

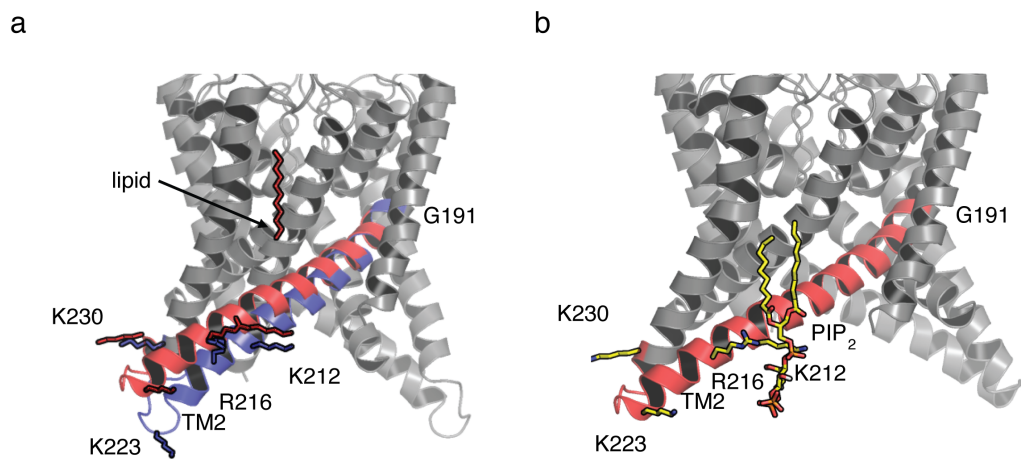


Figure 5.10: Comparison of the predicted PIP₂ binding site with the lipid binding site in the TREK-2 X-ray crystal structure (PDB id: 4BW5). (a) Overlay of the two alternative TREK-2 conformations, with co-crystallized lipid (red) in the 'up-state', and the 'down-state'. Predicted basic PIP₂-interacting residues on TM2 and TM3 are shown as sticks. In the 'up-state' TREK-2 structure, pivoting of TM2 at the absolutely conserved hinge glycine G191 results in an alternative upward movement of TM2-TM3 linker towards the membrane. (b) Modelling of PIP₂ into the lipid binding site revealed by the TREK-2 structure.

5.5 Docking TPenA in new TREK-2 structure

By now, two TREK-2 structures in alternative conformations exist ('down' and 'up' state, Figure 5.10a and Section 5.4.3). To test which one of the two new TREK-2 conformations better fits the functional mutagenesis data, ensemble dockings were repeated with TREK-1 models based on the 'down-state' of TREK-2 (Figure 5.11a) and the 'up-state' of TREK-2 (Figure 5.11b). The 'down-state'-based TREK-1 models (Figure 5.11b) displayed more (F185, L297) false positives than the 'up-state'-based models (F185). The 'up-state' is therefore more likely to resemble the TREK-1 conformation at which the *in vitro* mutagenesis data was obtained, when TREK-1 was gated open intracellularly by pH. Interestingly, the major differences between the two different TREK-1 models centre again on TM4. While TREK-1 models based on the 'up-state' exhibit no false positive on TM4, dockings of TPenA into 'down-state' models reveal TPenA interactions with residue L297 on TM4, contrary to the functional mutagenesis data.

To examine the influence of TM4 on the differences observed between the 'up' and 'down' state TREK-1 dockings, the C_{α} -RMSF was calculated for all residues in both 'up-state'-based models and 'down-state'-based TREK-1 models. This revealed that the two basic clusters that were predicted to interact with PIP₂ in coarse-grained simulations (the C-terminus of TM4, and the TM2-TM3 linker) are more flexible in the 'down-state' than in the 'up-state' models (Figure 5.12a). Based on this finding, one may speculate that specific lipid interactions may stabilise either the 'up-state' or the 'down state' as there appears to exist a stabilising effect on the TM2-TM3 linker and the C-terminal end of TM4.

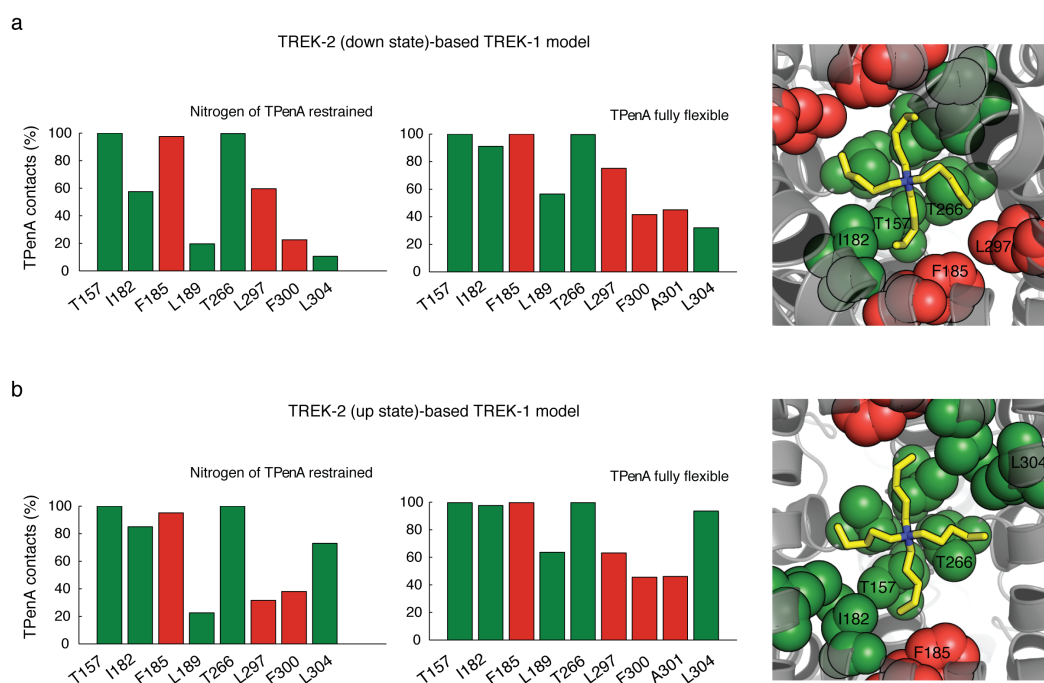


Figure 5.11: Ensemble docking of TPenA into TREK-1 models. TPenA docked into models based on **(a)** the lipid-bound TREK-2 structure ('up state') and **(b)** the alternative TREK-2 structure ('down state'). 'Hits' are shown in green, false positive interactions in red. The 'up state' exhibits the least number of false positives and correlates better with the functional mutagenesis data than the 'down state'.

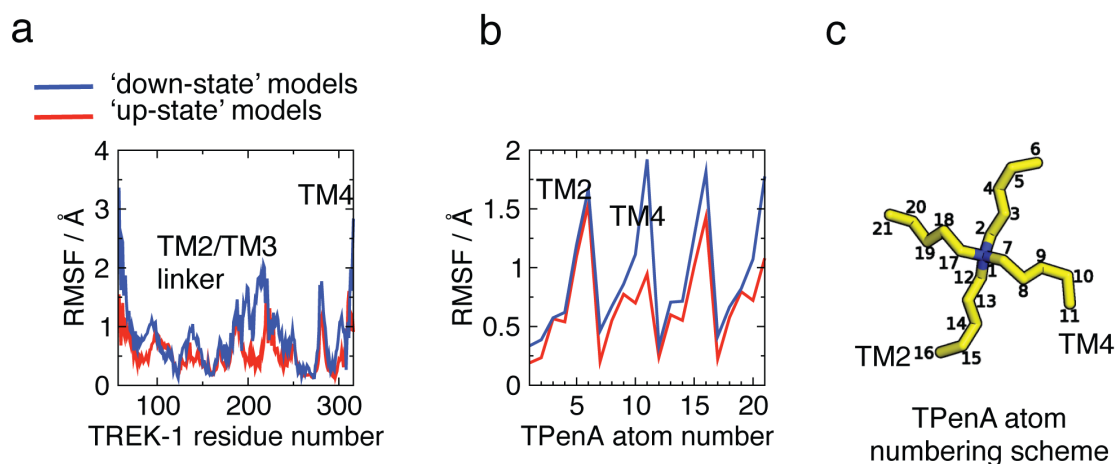


Figure 5.12: Conformational dynamics of TREK-2-based TREK-1 homology models. 'Down state' models (blue) are based on the alternative, preliminary TREK-2 structure, 'up state' models (red) on the lipid-bound TREK-2 structure. (a) RMSF of TREK-2-based homology models ($n = 400$). There is considerable flexibility in the 'down state' models, particularly in the C-terminal part of TM4 and in the TM2/TM3 linker, which exhibited large number of contacts with PIP₂ in CG simulations. (b) Conformational flexibility of TPenA in ensemble dockings. The central nitrogen atom of TPenA was restrained to the position found in the 2JK5-KcsA structure and the average RMSF for each TPenA atom calculated for dockings into the 'down-state' and 'up-state' models. The cavity below the selectivity filter, to which TPenA binds, is lined by helices TM2 and TM4. In 'down-state' models, the RMSF of TPenA, for those alkyl tails extending towards the cavity-lining residues of TM4, is up to 2x higher than in 'up state' models. This state-dependent difference in TPenA mobility reflects the high flexibility in TM4 in models based on the 'down-state' structure. (c) Atom numbering scheme for TPenA ligand. Alkyl tails facing TM2 and TM4 are marked.

However, this hypothesis needs to be evaluated more carefully by atomistic molecular dynamics simulations of TREK-1/lipid bound models, including models based on the final TREK-2 'down' structure.

Nonetheless, it is of interest to analyse the RMSF of the TPenA ligand docked into the cavity below the selectivity filter (Figure 5.12b). The flexibility of the central N atom (TPenA atom number '1', Figure 5.12c) is very low

as it is restrained to the position of the N atom in the bound QA ion of the 2JK5-KcsA crystal structure. With increasing distance from the central N atom, the RMSF of each TPenA atom increases. While the RMSF distribution among the C atoms of the alkyl tails is approximately equal (four-fold symmetric) in the 'down state'-based models (blue), it is decreased by factor two in 'up-state' based models, but only for alkyl tails pointing towards residues on TM4. This relates protein flexibility in the C-terminal part of TM4 to ligand flexibility near the selectivity filter. There exists ample experimental evidence that regulatory signals received at the C-terminus of TM4 are somehow transduced to gating at the selectivity filter [41, 80], but no specific mechanism has been proposed or directly observed in simulations, due to the lack of crystal structures and suitable homology models.

These findings provide support for the hypothesis that conformational fluctuations at the C-terminal end of TM4 are related to the flexibility of a ligand bound near the selectivity filter. They also report conformational fluctuations at the TM2-TM3 linker, which may be equally important for the flexibility of the TPenA binding site.

5.6 Discussion

Results from docking studies and the comparison between TREK-1 models based on various templates indicate that the ensemble docking approach, together with the comparison to functional mutagenesis data, provides a valuable tool for selection of the best template structure to model TREK-1. The ensemble docking method could distinguish between closely related templates and

between two templates of the same protein, but in alternate conformations. Moreover, the generated models could then be used to predict lipid binding sites in TREK-1. Nevertheless, the results presented here suffer from some limitations, which are discussed in some detail below.

It was argued that the most suitable template for modelling TREK-1 could be chosen by comparison of *in silico* dockings to functional mutagenesis data. This rationale presumed that cysteine mutations which inhibited TPenA binding *in vitro* corresponded to *in silico* interactions of the docked TPenA molecule with residues in the cavity of TREK-1. Several residues which affect TPenA inhibition upon mutation are unlikely to form part of the TPenA binding site but these residues may affect channel gating. Thus, a change in TPenA IC₅₀ may not be due to an (allosteric) change of the conformation of the binding site, but could be the result of movements at the helix bundle crossing that alter TPenA access to the pore. Nonetheless, the good fit of the closely related TREK-2 structures with the functional scanning mutagenesis data suggests that TPenA binds indeed at the predicted site. However, the variation between the 'up' and 'down' state TREK-2-based models also highlights the effect of alternative conformations of the TM helices on the TPenA blocker binding site.

Part of the structural differences between the TWIK-1- and TRAAK-based models are the degree of packing between TM2 and TM4, and its impact on the TPenA binding site. The more acute angle between TM4 and the bilayer normal in TRAAK-based models versus KvAP-based models reduces the likelihood of residues on TM4, which do not affect sensitivity to TPenA inhibition, to interact with TPenA *in silico* and may explain why TRAAK-based models correlate better with functional mutagenesis data than KvAP-based models. For example,

the orientation of TM4 in TRAAK-based models, in comparison to KvAP-based models, reduces the probability of L304 four-fold to interact with TPenA.

The predicted PIP₂ interactions sites are only a result of coarse-grained simulations. It is freely acknowledged that the specificity of anionic lipid interactions could not be studied in detail here. Accordingly, the results obtained here were restricted to qualitative observations and atomistic simulations are necessary and are carried out now using the new TREK-2 structures. Nevertheless, the PIP₂ binding observations were consistent with the previously determined C-terminal role of TM4 [146] and the lipid composition clearly has a substantial influence on the ability of PIP₂ to bind to TREK-1.

5.7 Summary

TREK-1 models were built, based on the recently determined structures of the homologous TRAAK, and TWIK-1 channels. *In silico* docking of QA into the pore of these new TREK-1 models confirmed that TPenA binds within the inner cavity just below the selectivity filter [34]. Furthermore, when compared to the functional scanning mutagenesis data obtained, the TRAAK-based model exhibited the least number of false positive interactions with TPenA, and is therefore likely to represent the best structural model of TREK-1 currently available. This is perhaps not surprising given the high degree of homology between TREK-1 and TRAAK, especially within the transmembrane-pore region [92, 321]. However, the observation that a TWIK-1-based model provides no better fit to the experimental data than any KvAP-based model highlights differences between the two pore-lining helices (TM2 and TM4) of TRAAK and TWIK-1.

Chapter 6

Conclusions

The general subject of the work presented in this thesis are K^+ ion channels, their mechanism of gating, and their interaction with lipids. K^+ ion channels play crucial roles in controlling electrical excitability and ion transport through cell membranes, and regulate diverse physiological processes such as salt and ion balance, heart rate, hormone secretion and sensory perception. This thesis has reported a range of simulations and computational experiments that probe the mechanisms by which K^+ channels gate, and investigated the molecular level interactions between K^+ channels and the lipid membrane. The motivation of performing such studies was to gain insight into the principles by which K^+ channels, which are key factors for cellular function, participate in fundamental biochemical processes, such as the binding to the secondary messenger lipid phosphatidylinositol-(4,5)-bisphosphate (PIP_2), and how these processes control K^+ channel gating. Comparing *in-silico* studies of K^+ channel gating to X-ray crystal structures required molecular dynamics (MD) simulations at the atomistic level. On the other hand, probing the interactions of membrane lipids such as PIP_2 with K^+ channels necessitated a multi-scale approach, due to the extended ($0.5 \mu s$) time-scale of the membrane lipid diffusion and binding event.

Throughout this thesis, there has been a trend moving from well-characterised Kir channels from bacteria (KirBac) to their more complex eukaryotic homologues. Eukaryotic Kir channels exhibit complex lipid regulation. The focus has then shifted toward the most recently cloned – and probably most exciting – group, K^+ channels of two pore domains (K2P).

The primary conclusions that can be drawn from the research work reported in this thesis are that a) Kir channels gate at the helix bundle crossing while TREK-1, the prototypical K2P channel, gates near the selectivity filter and b) multi-scale simulations can accurately characterize the binding site of PIP_2 to an ion channel, matching crystallographic predictions. The individual studies on the basis of which these two conclusions were drawn are briefly explained in the following two sections, mostly following the order by which they were presented in the preceding results chapters.

6.1 K^+ channel gating

The primary gate of Kir channels was believed to be at the helix bundle crossing. However, a series of KirBac structures proposed that changes in the cytoplasmic domain (CTD) are directly coupled to the conductivity status at the selectivity filter [64]. The conformational changes that control opening of KirBac3.1, a bacterial homolog to Kir channels, at the helix bundle crossing, could be captured using MD simulations. In the mutant S129R structure, the side chains of the S129R mutation effectively blocked the ion permeation pathway at the helix bundle crossing. However, in Chapter 3, *in-silico* mutation of R129 to the wild-type serine clearly resulted in a structural model that was open at the helix

bundle crossing. Further support for the hypothesis that Kir channels gate at the helix bundle crossing was provided by the MD simulations of this open model, which revealed closing at the helix bundle crossing, if R129 was converted back to the wild-type serine. The observed opening of the helix bundle in simulations where R129 was not converted back to serine, or where R129 was mutated to aspartate, provided further evidence for an active role of the helix bundle in gating. Although gating may also occur at the selectivity filter and affect channel conductivity, the helix bundle clearly needs to be open for K^+ to permeate through the ion channel.

Secondly, MD simulations in Chapter 3 suggested that activatory mutations in the KirBac cytoplasmic domain increase channel permeability by stabilising an electrostatically favorable open conformation. Although effects of the KirBac3.1 cytoplasmic activatory mutation S205L on the kinetics of channel gating cannot be excluded, it is likely that changes in the electrostatic properties of the cytoplasmic pore contribute to the activatory effect of this mutation located at the cytoplasmic subunit interfaces. Structural changes at these interfaces clearly change the electrostatic potential within the cytoplasmic pore and the increased ability to concentrate ions in the cytoplasmic pore might facilitate ion conduction through the channel. As the structural changes evoked by the activatory mutation in the cytoplasmic domain are quite subtle, this would explain why the mechanism by which mutations in the subunit interfaces lead to activation was found to be primarily of an electrostatic nature.

Together, these observations point towards a model of Kir channel gating by which the cooperative effect of changes in the electrostatic pore profile, coupled with conformational transitions at the helix bundle crossing lead to opening or

closing of the channel. In fact, further evidence for this model was provided in Chapter 4, where a helical C-linker was found to stabilise the helix bundle in an open conformation. This is of particular note, as it reinforces the idea that PIP₂, upon binding to the C-linker induces a conformation that would lower the energy barrier to activation. Another interesting observation that emerged from the studies of Kir channel gating in Chapter 4 is that the Kir channel pore electrostatics are sensitive to the C-linker/PIP₂ binding site conformation. As this electrostatic mechanism is similar to what has been observed in the CTD pore of the KirBac3.1 activatory mutant S205L, this may be a conserved principle of gating, that rather subtle structural changes alter the electrostatic energy profile, thereby contributing to an increased pore permeability.

To explore the mechanism of gating in K2P channels, several structural models of TREK-1 were built in Chapter 5, based upon the recent crystal structures of the related K2P channels, TWIK-1 and TRAAK. These two different structural models of TREK-1 were validated by comparison to functional scanning mutagenesis data, which revealed that TRAAK provides the best structural template for modelling of TREK-1. This new structural model of TREK-1 was found to be open at the helix bundle crossing, and together with the functional data by Rapedius et al [316], indicates a mechanism of gating at or near the selectivity filter. A crystal structure of TREK-1 was deposited in the Protein Data Bank by Carpenter *et al.* in the final month of this PhD project. Therefore, MD simulations on this structure are being carried out by members of the Sansom lab to further investigate the mechanism of K2P channel gating and are interpreted in the context of the studies described in this thesis.

6.2 K^+ channel/lipid interactions

While the first theme of this thesis focused on mechanisms of K^+ channel gating, the second aspect of the work reported here concerns K^+ channel/lipid interactions in a physiological bilayer, in particular interactions of K^+ channels with modulatory lipids, such as the secondary messenger PIP_2 , which activates all Kir channels and regulates several other K^+ channels [322].

A multi-scale approach was used, consisting of sequential coarse-grained and atomistic (MD) simulations of Kir2.2 embedded in a phospholipid/ PIP_2 bilayer, to determine whether this computational approach leads to the same PIP_2 binding site as observed in the PIP_2 -bound Kir2.2 crystal structure. The PIP_2 binding observed in simulations in Chapter 4 correctly predicted the PIP_2 binding site in Kir2.2 even when slightly different structures were used as starting coordinates for these simulations. These results therefore demonstrate the predictive power of this computational approach for the study of protein interactions with PIP_2 and possibly other modulatory lipids. Consequently, this method may now be confidently applied to the prediction of novel PIP_2 binding sites as well as the exploration of other lipid/channel interactions. For example, several eukaryotic voltage-gated potassium channels are regulated by PIP_2 [121], and enough high-resolution crystal structures now exist to enable similar multi-scale simulations of PIP_2 binding to these structures. Also, cholesterol has been shown to directly regulate Kir channel function but its precise binding site is unknown [270]. Many other unrelated ion channels and transporters, as well other classes of membrane proteins, have also been shown to be regulated by lipids [323, 324].

Having validated this multi-scale approach, it was then applied to study both Kir (Chapter 4) and K2P (Chapter 5) channels with PIP₂ in more physiological bilayers, that included anionic phospholipids in the inner leaflet. The anionic phospholipid phosphatidylserine (PS) attenuated PIP₂ binding to both TREK-1 and Kir2.2, providing evidence for an electrostatic attraction contributing to ion channel/PIP₂ interactions. Interestingly, the observed binding of PS to Kir2.2 indicated a secondary binding site for PS. The idea of a secondary binding site for anionic lipids in Kir channels has not been unprecedented [140], and the observed interactions of PS with K220 on the CD loop is in line with recent computational predictions by D'Avanzo *et al.* [292].

PIP₂ was also found to bind to some of the functionally predicted TREK-1 residues [99], although this part of the protein (C-terminal end of last TM4) was modelled *de novo* as helix, following sequence-based prediction [325], and may therefore not be representative of a true physiological conformation. Nevertheless, in addition to the prediction of the known PIP₂-sensitive residues on the C-terminus, Chapter 5 predicted a second PIP₂ binding site in TREK-1, located between TM2 and TM3, which strongly suggests that TREK-1 exhibits a high affinity for anionic lipids. Interestingly, electron density for a lipid tail that was found in the new TREK-1 structure by the Carpenter group would be compatible with a PIP₂ molecule bound to the second predicted PIP₂ binding site.

6.3 Methodological advancements

To study K2P channels, for which a paucity of structural data was available at the outset of this thesis, an improved method of homology modelling had to be developed. Docking of quaternary ammonium (QA) ions into TREK-1 homology models and comparison of the predicted binding site to functional mutagenesis was the approach taken by Piechotta *et al.* to arrive at a homology model of TREK-1 [34]. However, application of their docking method, which treated the homology model as rigid, or which allowed only limited side chain flexibility, to TREK-1 models based on TRAAK and TWIK, did not lead to reproducible docking results in this thesis (Chapter 5). This was presumably because the templates used to construct homology models of TREK-1 in Chapter 5 were too closely related to each other. Also, an arbitrary choice had to be made in Chapter 5 as to which of the many MODELLER-generated homology models per template to choose for these dockings. The comparison of these dockings between models based on different templates might therefore be biased, as these templates are very closely related.

Therefore, rather than docking QA ions into an arbitrarily chosen homology model, QA ions were docked into an *ensemble* of homology models, assuming that the obtained average would be more informative than a docking calculation based on a single structure. Although it is well-known that MODELLER leads to an ensemble of structures that all satisfy certain spatial restraints, it is not obvious to use MODELLER to create an ensemble to be used for docking calculations. Instead, the output of docking calculations could be used as input for MODELLER, to further ‘minimize’ the system. Alternatively, the docking

parameters could be optimised, in the hope to obtain better convergence and reproducibility of the docking result. Thus, this novel modelling-docking approach has solved the objective technical problem of predicting the binding site of QAs in different TREK-1 models based on closely related templates. Based on that prediction, it is now possible to select the template that leads to models whose binding site fits the functional mutagenesis data best and on which to build a final structural model of TREK-1. This technique has now been shared and used by C. Nichols' group at the Washington University, St. Louis, USA [292] and by our collaborators at Pfizer/Neusentis, Cambridge, UK.

6.4 Further work

Considering the asymmetry of the TREK-1 channel pore structure, and given the method to predict the QA binding site in TREK-1 channels, this modelling-docking technique might now alternatively be applied to investigate the binding of more complex small molecules to K2P channels. An obvious choice would be to dock asymmetric QA ions into asymmetric K2P pores, which could reveal potential binding sites for drug-like molecules, specific inhibitors or activators. A series of docking studies with TREK-1 models and asymmetric QA derivatives, with two pentyl, and two ethyl tails for example, could indicate whether asymmetric pore blockers would have a higher inhibitory potency in TREK-1, compared to symmetric tetraethylammonium, which does not inhibit TREK-1. If this approach was to be repeated in a closed and open TREK-1 structure, one could speculate about designing an asymmetric small molecule that binds to either the open or the closed structure, with the aim to stabilise either

the open or closed state. The fenestrations which are seen in TREK-1, TRAAK, TWIK-1, and other voltage-gated ion channels [29] are an example of state-dependent degree of pore asymmetry that could be exploited to this purpose. An additional experiment would be to dock QA derivatives into a model of the functional TASK-1/TASK-3 heterodimer which could be interesting to design specific blockers of tetrameric channels consisting of four different subunits. Tentatively, one might hypothesise that the degree of asymmetry, ranging from symmetric Kir, over TREK-1, to TASK-1/TASK-3, would determine the asymmetry of the most potently inhibiting QA molecule. Other small molecules of interest are BL-1249, which activates TREK-1, but inhibits TRESK, and fluoxetine. In summary, there are several potentially interesting applications in which this modelling-docking technique could be exploited, and, of course, the method also needs rigorous statistical assessment and further improvement.

The validated multi-scale lipid-binding prediction can now also be used to predict the binding site of several natural occurring lipid-like molecules, such as capsaicin, sanshool or spilanthol. At present, it is not clear whether sanshool, which evokes a tingling sensation distinct from capsaicin binds to the same receptor as capsaicin, the TRPV channel, or, as suggested, to K2P channels.

In short, there exist at least two avenues by which the techniques presented in this thesis could be further exploited. First, to study lipid/ion channel interactions using the multi-scale method and using increasingly complex physiological bilayers, including, for example, exogenous lipophilic compounds, or endogenous lipids such as cholesterol, lysophosphatidic acid, or arachidonic acid. Secondly, combined homology modelling and docking approaches could be used to study the interaction of K^+ channels with small molecules that are

of interest in a drug discovery context. Either way, the complex interaction of K^+ channels with physiological bilayers, secondary messengers, regulatory and cellular scaffolding proteins, is likely to require a combination of computational approaches, from coarse-grained, multi-scale to atomistic simulations, and small-molecule docking calculations.

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List of abbreviations

Roman Symbols

A	Microscopic property of an arbitrary state
$\bar{A}$	Time-average of property A
$\langle A \rangle$	Ensemble-average of property A
A_{ij}, B_{ij}	AutoDock parameters for van der Waals interactions
b_{ij}	Equilibrium bond length between i-th and j-th particle ($\text{\AA}$)
c_i^∞	Bulk density at distance far away from the protein ($\text{C \AA}^{-3}$)
C_{ij}	Lennard Jones coefficients
C'_{ij}, D_{ij}	AutoDock parameters for hydrogen potential
$[\text{Cl}^-]_i$	Intracellular concentration of Cl^- ions (M)
$[\text{Cl}^-]_o$	Extracellular concentration of Cl^- ions (M)
C_n	Dihedral constant in Ryckaert-Belleman potential (kJ mol^{-1})
C_v	Heat capacity at constant volume ($\text{kJ mol}^{-1} \text{K}^{-1}$)

List of abbreviations

E	Energy of a multi-body particle system with Hamiltonian H (kJ mol ⁻¹)
E_{kin}	Kinetic energy (kJ mol ⁻¹)
$E(\theta)$	AutoDock factor reflecting deviations from optimum hydrogen bond geometry
F	Force acting on atom with mass m (kJ mol ⁻¹ nm ⁻¹)
F	Faraday constant (C mol ⁻¹)
ΔG	Gibbs free energy (kJ mol ⁻¹)
$\Delta G_{el}(z)$	Electrostatic energy of ion binding to the pore axis of a K ⁺ channel (kJ mol ⁻¹)
ΔG_{el}^I	Electrostatic energy of an ion in the bulk (kJ mol ⁻¹)
ΔG_{el}^P	Electrostatic self-energy of a protein (kJ mol ⁻¹)
ΔG_{el}^{P-I}	Electrostatic energy of a protein-ion complex (kJ mol ⁻¹)
H	Hamiltonian energy function of a particle system
I	Macroscopic current (A)
k_B	Boltzmann constant (kJ K ⁻¹)
$[K^+]_i$	Intracellular concentration of K ⁺ ions (M)
k_{ij}	Bond force constant between i-th and j-th particle (kJ mol ⁻¹ nm ⁻²)

List of abbreviations

$\tilde{k}_{ij}^b$	Fourth power bond force constant between i-th and j-th particle (kJ mol ⁻¹ nm ⁻⁴)
k_{ijkl}^ϕ	Dihedral force constant (kJ mol ⁻¹)
k_{ijk}^θ	Force constant of angular potential (kJ mol ⁻¹ rad ⁻²)
k_{ijkl}^ξ	Improper dihedral force constant (kJ mol ⁻¹ rad ⁻²)
$[K^+]_o$	Extracellular concentration of K ⁺ ions (M)
k_{pr}	Force constant of harmonic restraint potential (kJ mol ⁻¹ nm ⁻²)
l	Length of box size (Å)
m	Mass of an arbitrary particle (kg)
N	Number of ion channels
n	Multiplicity in dihedral potential
$[Na^+]_i$	Intracellular concentration of Na ⁺ ions (M)
$[Na^+]_o$	Extracellular concentration of Na ⁺ ions (M)
N_c	Number of constraints of a N -particle system
N_{com}	Number constraints taking account of the center-of-mass
N	Number of atoms in a multi-body particle system
P_o	Single channel open probability
P_0	Reference pressure (bar)

$\mathbf{p}$	Momentum of a particle of a multi-body system
P_{Cl}	Relative permeability to Cl^-
$\mathbf{p}_i(t)$	Momentum of the i-th particle at time t
P_K	Relative permeability to K^+
P_{Na}	Relative permeability to Na^+
P	Pressure in a multi-body particle system (bar)
q_i	Charge of i-th particle (C)
q_j	Charge of j-th particle (C)
$\mathbf{R}$	Position of the nucleus of an atom with wave function Ψ
R	Rydberg constant ($\text{J mol}^{-1} \text{K}^{-1}$)
r	Radius ($\text{\AA}$)
$\mathbf{r}_0$	Reference position of a particle of a multi-body system
$\mathbf{r}$	Position of a particle of a multi-body system
$\mathbf{r}_{el}$	Position of the electrons of an atom with wave function Ψ
$\mathbf{r}_i(t)$	Position of the i-th particle at time t
r_{ij}	Distance between i-th and j-th particle ($\text{\AA}$)
$\mathbf{r}_j(t)$	Position of the j-th particle at time t
$\mathbf{r}_k(t)$	Position of the k-th particle at time t

List of abbreviations

$\mathbf{r}_l(t)$	Position of the l -th particle at time t
ΔS_{conf}	Loss in entropy upon ligand binding ($\text{kJ mol}^{-1} \text{K}^{-1}$)
S_i, S_j	AutoDock solvation parameters
Δt	Time step (s)
T	Temperature (K)
t	Time (s)
T_0	Reference temperature (K)
V	Effective potential of a many-body particle system (kJ mol^{-1})
V_{angle}	Angle potential (kJ mol^{-1})
V_b	Potential for bonded interactions (kJ mol^{-1})
V_{bond}	Covalent bond length potential (kJ mol^{-1})
V_C	Coulomb potential (kJ mol^{-1})
$V_{improper}$	Improper dihedral potential (kJ mol^{-1})
V_K	Equilibrium potential for K^+ (V)
V_{LJ}	Lennard-Jones potential (kJ mol^{-1})
V_{bound}^{L-L}	Self-energy of the AutoDock ligand bound to a protein (kJ mol^{-1})
$V_{unbound}^{L-L}$	Self-energy of the unbound AutoDock ligand (kJ mol^{-1})

List of abbreviations

V_{bound}^{L-P}	Self-energy of the AutoDock ligand-protein system in the ligand-bound state (kJ mol ⁻¹)
$V_{unbound}^{L-P}$	Self-energy of the AutoDock ligand-protein system in the ligand-unbound state (kJ mol ⁻¹)
V_m	Transmembrane voltage (V)
V_{nb}	Potential for non-bonded interactions (kJ mol ⁻¹)
V_{bound}^{P-P}	Self-energy of the AutoDock protein in the ligand-bound state (kJ mol ⁻¹)
$V_{unbound}^{P-P}$	Self-energy of the AutoDock protein in the ligand-unbound state (kJ mol ⁻¹)
V_{pr}	Harmonic restraint potential (kJ mol ⁻¹)
V_{rest}	Resting membrane potential (V)
$V_{torsion}$	Dihedral potential (kJ mol ⁻¹)
V	Volume of a multi-body particle system (Å ³)
W_{elec}	AutoDock electrostatics weighing factor
W_{hbond}	AutoDock hydrogen bond weighing factor
W_{sol}	AutoDock solvation weighing factor
W_{vdw}	AutoDock van der Waals weighing factor
Z	Valence of the permeant ion

Z_i Valency of the i -th ion species

Greek Symbols

β Boltzmann factor (kJ^{-1})

β_{ij} Compressibility tensor (bar^{-1})

δ_{ij} Kronercker-Delta symbol

ϵ Relative dielectricity

ϵ_0 Dielectricity constant ($\text{C V}^{-1} \text{ m}^{-1}$)

ϵ_{ij} Depth of potential wall for pairwise potential V_{LJ} between i -th and j -th particle (kJ mol^{-1})

ϵ_p Relative dielectricity of protein ($\epsilon_p \approx 10$)

ϵ_w Relative dielectricity of water ($\epsilon_w \approx 78$)

γ Unitary conductance (S)

κ Compressibility (bar^{-1})

λ Velocity rescaling factor

μ Chemical potential (kJ mol^{-1})

μ_{ij} Parrinello-Rahman barostat scaling matrix

ν Number of degrees of freedom of N -particle system

Ψ Wave function of an atom with mass m

List of abbreviations

ϕ	Wave function of the electrons
Φ	Wave function of the nucleus
$\phi_{0,ijkl}$	Equilibrium angle between the two normal vectors of planes i, j, k and j, k, l (rad)
ϕ_{ijkl}	Angle between the two normal vectors of planes i, j, k and j, k, l (rad)
ρ	Phase space density
ρ_f	Fixed charge density for proteins ($\text{C } \text{\AA}^{-3}$)
ρ_m	Mobile charge density for ions in solutions ($\text{C } \text{\AA}^{-3}$)
ρ_{tot}	Total charge density including fixed charge and mobile charge density ($\text{C } \text{\AA}^{-3}$)
σ_{sol}	AutoDock solvation parameter smoothing factor ($\text{\AA}$)
σ_{ij}	Distance at which the potential energy is zero for the pairwise potential V_{LJ} between the i -th and j -th particle ($\text{\AA}$)
$\mathcal{T}$	Time interval for forming the time average of property A (ps)
τ	Berendsen time constant (ps)
τ_p	Pressure coupling constant (ps)
τ_T	Velocity rescaling constant in Berendsen thermostat (ps)
$\theta_{0,ijk}$	Equilibrium bond angle between i -th, j -th, and k -th particle (rad)

List of abbreviations

θ_{ijk}	Bond angle between i-th, j-th, and k-th particle (rad)
τ_P	Pressure coupling constant (ps)
τ_T	Temperature coupling constant (ps)
$\chi(\mathbf{r})$	Scaling term prohibiting mobile ion positions to overlap with fixed protein charges
$\xi_{0,ijkl}$	Improper dihedral equilibrium angle (rad)
ξ_{ijkl}	Improper dihedral angle (rad)
$\mathcal{Z}$	Partition sum of particle system with Hamiltonian H

Acronyms

AA	Arachidonic acid
AMBER	Assisted Model Building with Energy Refinement
APBS	Advanced Poisson Boltzmann Solver
AT	Atomistic
ATP	Adenosine triphosphate
BL-1249	[(5,6,7,8-tetrahydro-naphtalen-1-yl)-[2-(1H-tetrazol-5-yl)-phenyl]-amine]
Ca^{2+}	Calcium ion, intracellular concentration $[\text{Ca}^{2+}]_i$: 10^{-7} mM, extracellular concentration $[\text{Ca}^{2+}]_o$: 2.25–2.52 mM
cAMP	Cyclic adenosine monophosphate

List of abbreviations

CG	Coarse-grained
CHARMM	Chemistry at Harvard Molecular Mechanics
Cl ⁻	Chloride ion, [Cl ⁻] _i : 2.5–50 mM, [Cl ⁻] _o : 115 mM
CTD	C-terminal domain (intracellular/cytoplasmic assembly)
DAG	Diacylglycerol
G(q)PCR	G(q)-protein coupled receptor
GHK	Goldman-Hodgkin-Katz equation
GROMACS	Groningen Machine for Chemical Simulation package
GROMOS	Groningen Molecular Simulation
HBC	Helix bundle crossing
IC ₅₀	Inhibitory potency
IUPHAR	International Union of Basic and Clinical Pharmacology
K2P	K ⁺ channel of two pore domains
K ⁺	Potassium ion, [K ⁺] _i : 140 mM, [K ⁺] _o : 3.5–5 mM
KcsA	Prokaryotic K ⁺ channel from bacteria <i>Streptomyces lividans</i>
Kir	Inwardly rectifying K ⁺ channel
KirBac	Inwardly rectifying bacterial K ⁺ channel, homolog to Kir
Kv	Voltage-gated K ⁺ channel

List of abbreviations

KvAP	Kv channel from archeabacterium <i>Aeropyrum Pernix</i>
l-BFGS	limited-memory Broyden-Fletcher-Goldfarb-Shanno quasi-Newtonian minimizer
LINCS	Linear constraint solver
LPC	Lysophospholipids
MD	Molecular dynamics
MlotiK	Cyanc nucleotide-regulated K ⁺ -channel from <i>Mesorhizobium loti</i>
MscL	Mechanosensitive cationic large conductance channel
MthK	Ca ²⁺ -activated channel from the archaeon <i>Methanobacterium thermoautotrophicum</i> ; comparison with KcsA channel lead to K ⁺ channel gating hypothesis
MTS-TBAO	8-(tributylammonium)octyl methanethiosulfonate
MTSET	2-(trimethylammonium)ethyl methanethiosulfonate
Na ⁺	Sodium ion, [Na ⁺] _i : 12 mM, [Na ⁺] _o : 135–145 mM
NaK	Non-selective bacterial cation channel
NFAT	Nuclear factor of activated T-cells
OPLS	Optimised Potentials for Liquid Simulations
PIP ₂	Phosphatidylinositol-(4,5)-bisphosphate
PA	Phosphatidic acid

List of abbreviations

PB	Poisson-Boltzmann
PC	Phosphatidylcholine
PDB	Protein Data Base
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI(3,4,5)P	Phosphatidylinositol-3,4,5-trisphosphate
PI(4)P	Phosphatidylinositol-4-phosphate
PI(5)P	Phosphatidylinositol-5-phosphate
PI(3,5)P	Phosphatidylinositol-3,5-phosphate
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PIP	Phosphatidylinositolphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PME	Particle Mesh Ewald summation
POPC	Palmitoyl-oleoyl phosphatidylcholine
POPS	Palmitoyl-oleoyl phosphatidylserine
PPA	Polyphosphatidic acid

List of abbreviations

Prozac	Fluoxetine
PS	Phosphatidylserine
PSICOV	Precise structural contact prediction using sparse inverse covariance estimation
PUFA	Polyunsaturated fatty acids
QA	Quaternary ammonium ion
QM/MM	Quantum mechanical/molecular mechanics simulations
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
S1, S2, S3, S4	Ion positions along selectivity filter
scPIP ₂	Short-chain PIP ₂
SPC	Single Point Charge water model
TASK	TWIK-related acid-sensitive K ⁺ channel
TButA	Tetrabutylammonium
TEthA	Tetraethylammonium (also: TEA)
THepA	Tetraheptylammonium
THexA	Tetrahexylammonium
TM	Transmembrane helix, e.g. TM2 or TM4

List of abbreviations

TMD	Transmembrane domain
TPenA	Tetrapentylammonium
TProA	Tetrapropylammonium
TRAAK	TWIK-related arachidonic acid activated K ⁺ channel
TREK	TWIK-related K ⁺ channel
TRESK	TWIK-related spinal cord K ⁺ channel
TRP	Transient receptor potential channel
TWIK	Two pore domain weakly inward-rectifying K ⁺ channel
vdW	van der Waals