The influence of lipids on a potassium channel

–

KcsA unraveled

De invloed van lipiden op een kaliumkanaal

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KcsA ontrafeld

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
woensdag 24 november 2010 des middags te 2.30 uur

door

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geboren op *20 oktober 1980 te Apeldoorn*

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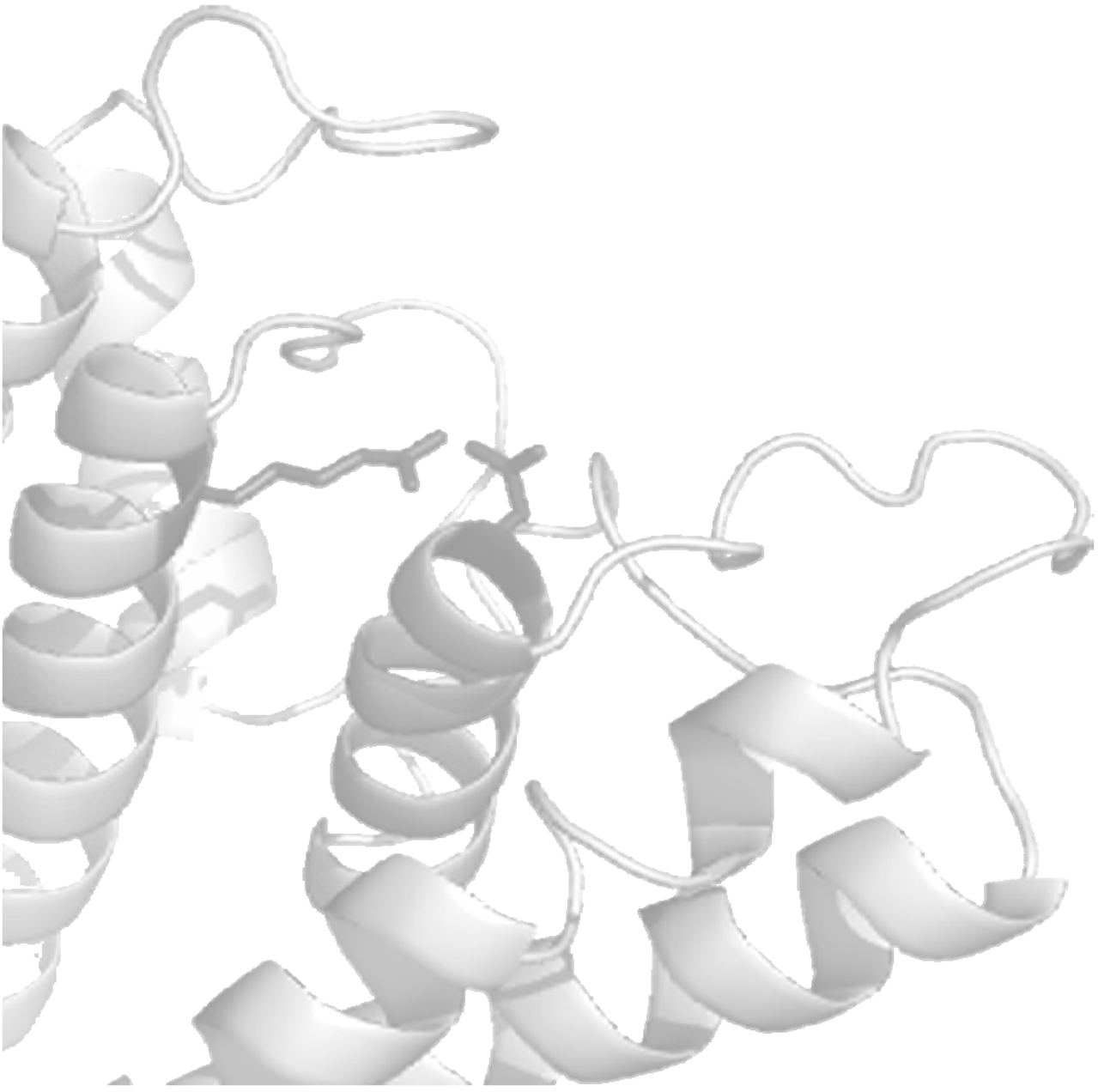
Dit proefschrift werd mogelijk gemaakt met financiële steun van NWO alsmede een travelgrant van de European Biophysical Societies' Association.

If we knew what it was we were doing,
it would not be called research,
would it?

Albert Einstein



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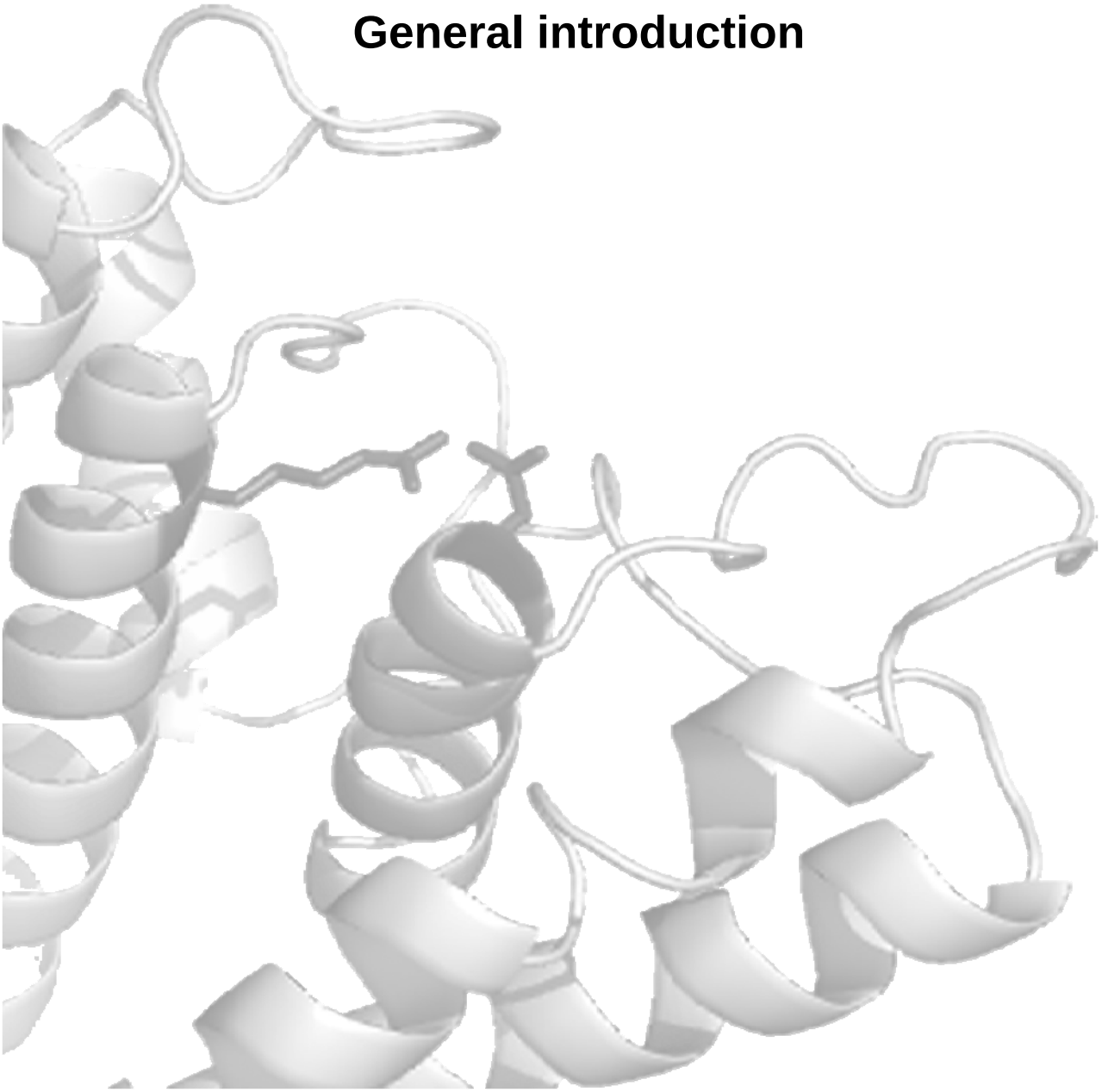
Chapter 1

General introduction



Membranes

All living organisms consist of one or more cells. Each cell is surrounded by a membrane. Membranes form a barrier between the inside of the cell and the exterior environment. This barrier prevents molecules inside the cell from leaking out but also forms an obstacle against the entering of unwanted molecules from outside the cell. Besides forming a barrier, membranes are also the means by which the cell communicates with the outside world via a wide variety of processes, e.g. transport of ions or molecules or via conformational changes of membrane components.

All membranes are composed of a combination of lipids and proteins which are held together by non-covalent interactions. Lipids are amphipathic molecules consisting of a hydrophilic headgroup and hydrophobic tails which are arranged in a double layer (bilayer) [1]. The hydrophobic tails of the lipids cluster together whereas the hydrophobic headgroups are in contact with the aqueous environment. This lipid bilayer is not a simple two dimensional plane in which lipids and protein components randomly diffuse as believed in earlier days [2]. Membranes are more complex structures which have a non-random distribution of proteins and lipid introducing patches of a distinct composition [3-5]. In addition, asymmetry between the leaflets within the bilayers occurs in many membranes [6, 7]. Furthermore, despite their common general architecture, membranes differ to great extent in their lipid and protein composition between different cell types and subcellular compartments [8, 9].

Protein-lipid interactions within the membrane form the main topic of this thesis. More specifically we want to know how lipids influence the assembly, stability and function of membrane proteins. To investigate these questions we make use of the bacterium *E.coli* and artificial membranes as an environment in which we look at the stability and function of the tetrameric bacterial potassium channel KcsA. Potassium channels belong to the class of the oligomeric membrane proteins, meaning that these proteins consist of two or more protein subunits assembled in a complex for function. First the general characteristics of lipids, membrane proteins and lipid-protein interactions will be discussed, followed by the common features of potassium channels in general and KcsA in particular.

Lipids

Membranes consist of many different lipids. Three main groups are glycerophospholipids, sphingolipids and sterols. This thesis will mainly focus on the glycerophospholipids which are composed of a glycerol backbone to which one or two hydrophobic fatty acids are attached via ester or ether bonds. The third hydroxyl group is attached to a phosphate moiety carrying an alcohol, which together form the lipid headgroup. Many different types of headgroups exist, which differ in e.g. charge, size, shape and their capacity to form hydrogen bonds. *Fig. 1* shows the general structure of glycerolipids with different headgroups, as they are used for the research described in this thesis.

It has been found that lipids can influence (oligomeric)membrane proteins in several ways [8-13] as will be discussed later in more detail. Therefore it seems that lipids play a significant role in protein structure and function, and thus in cell regulation. The involvement in these fundamental

processes makes protein-lipid interactions an important and attractive research topic, which in general is still poorly understood.

The bacterium *E.coli*, forms an attractive model to study protein-lipid interactions for several reasons. First of all it grows fast and is well studied. Moreover its entire genome is sequenced and it is easy to add extra genes by plasmid-based transformation. *E.coli* are protected from the outside environment by an inner and outer membrane with a peptidoglycan layer in between for cell stability. For our research it is of key importance that the *E.coli* inner membrane, in which our model protein KcsA resides, is relatively simple of composition. It is comprised of only a few different classes of lipids. About 75% of the membrane consists of the zwitterionic lipid phosphatidylethanolamine (PE), 20% of phosphatidylglycerol (PG), 5% of cardiolipin (CL) and trace amounts of phophatidic acid (PA) are present. In our *in vitro* studies additional to these *E.coli* lipids we make use of the zwitterionic phosphatidylcholine (PC) as a reference lipid. PG, CL and PA carry a negative charge under physiological pH. These classes of lipids perform a broad variety of functions in the *E.coli* membrane e.g. maintaining membrane fluidity and signaling. In addition, lipids influence membrane proteins in different ways depending on their specific properties as will be discussed later [14-17].

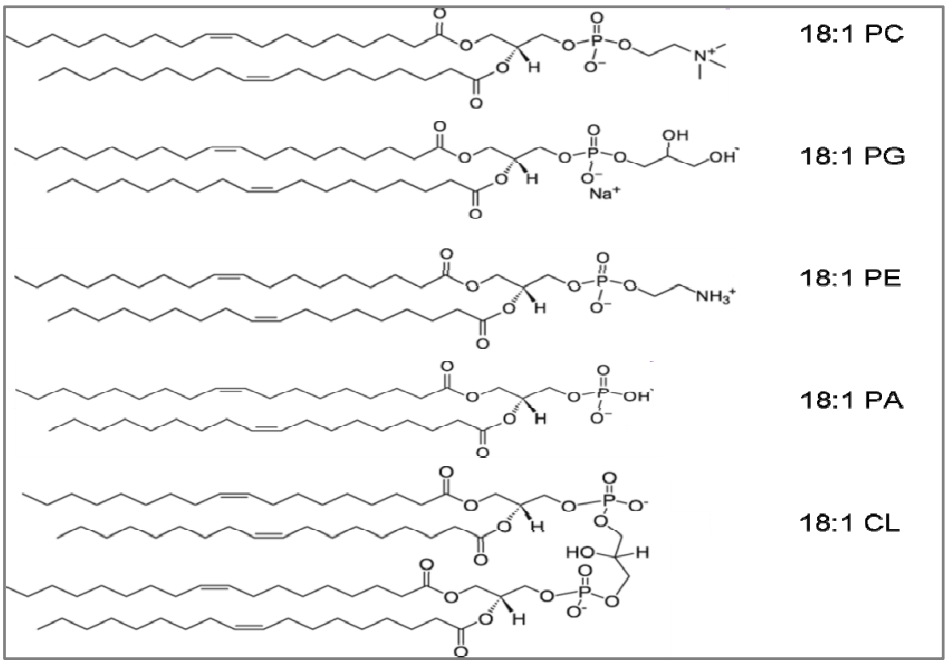


Figure 1: Chemical structure of different glycerophospholipids that are used in the research described in this thesis. Shown from top to bottom: phosphatidylcholine (PC) phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA) and cardiolipin (CL). All lipids have oleic acid tails and hence both acyl chains contain 18 carbon atoms and one double bond.

Membrane proteins

Membranes contain a fair amount of proteins, although the actual amount may vary based on the cell type, the type of organelle, the function of the particular membrane, and environmental conditions. The importance of membrane proteins is clear from their abundance. Between twenty and thirty percent of all open reading frames code for proteins that reside in the membrane [18]. Membrane proteins are often implicated in diseases, thereby forming an interesting target for drugs [19, 20]. Moreover, among protein domains, membrane interacting domains are found to be very prominent [21].

Proteins can interact with the membrane in several different ways. Integral membrane proteins span the entire lipid bilayer with stretches of hydrophobic amino acids. For inner membrane proteins these transmembrane segments most often have an α-helical structure which protects the polar groups of the peptide backbone by internal hydrogen bonding, thereby exposing the hydrophobic amino acids to the environment. Also polar and charged amino acids are found in these transmembrane helices, although their presence has to be compensated by the presence of sufficient hydrophobic residues [22]. At the membrane interface these transmembrane helices are often flanked by aromatic residues such as tryptophans [23], where they most likely function as membrane anchors [24-26]. Additionally positive charges are frequently present on the intracellular side of integral membrane proteins, which is known as the “positive inside rule” [27].

In the outer membrane of bacteria these integral membrane proteins often have a β-barrel structure, also shielding the polar residues from the hydrophobic interior of the membrane. Besides integral membrane proteins also peripheral membrane proteins are abundant. These proteins associate with the membrane interface by electrostatic or hydrophobic interactions rather than spanning the membrane.

This thesis will focus on the potassium channel KcsA which belongs to the class of the α-helical integral oligomeric membrane proteins.

Lipid-protein interactions

The structure as well as function of membrane proteins can be sensitive towards the membrane environment. For example, it is known that the presence of specific lipid species can affect the activity and stability of membrane protein complexes [28]. Additionally lipids can play a role in protein insertion, topogenesis and folding [29, 30]. Furthermore tightly bound lipids are often found in protein crystal structures, suggesting an important and specific interaction [11-13, 31]. Also the biogenesis of proteins is influenced by lipids in several ways. For example, the activity of the preprotein translocase complex depends on the surrounding lipid environment [9, 32], but also proteins that do not use the SecYEG complex for their insertion are dependent on the composition of the acceptor membrane [33]. After protein insertion the presence of specific lipids might influence the folding of a protein [30] or be involved in the assembly of oligomeric protein complexes [33].

One of the most well studied examples of an integral membrane protein that is influenced in multiple ways by its lipid environment is the potassium channel KcsA from the bacterium *Streptomyces lividans (Fig. 3 and 4).* In this

thesis we will use KcsA and several KcsA mutants to investigate the influence of lipids on tetramer assembly, tetramer stability and functional behavior. Before discussing these lipid influences in more detail, first some general information on potassium channels will be given.

Potassium channels

Potassium channels are found in virtually all types of cells and organisms, were they are involved in a multitude of physiological functions. They are tetrameric integral membrane proteins forming transmembrane aqueous pores through which potassium specifically permeates. Many molecular subfamilies of potassium channels are known, and these roughly correspond to the physiological signals by which pore opening is controlled e.g. calcium, pH and voltage. Most channels are found in the plasma membrane. They do not have a random and equal distribution in the membrane and are known to be able to form clusters [34, 35].

In general two classes of potassium channels are defined, the six-transmembrane-helix voltage-gated (Kv) and the two-transmembrane-helix inward-rectifier (Kir) subtypes [36-38] *(Fig. 2).* Voltage gated channels are activated by changes in electrical potential difference near the channel; these types of ion channels are especially critical in neurons, but are common in many cell types. Inward rectifier channels pass currents more easily in the inward direction than to the outside of the cell, helping to establish the resting membrane potential. All potassium channels have a “signature sequence” between the two most C-terminal transmembrane helices [39]. This sequence is responsible for the selectivity towards potassium and reads with minor variations: TMxTVGYG as shown by Shealy et al [40]. The alignment of this signature sequence in the tetrameric structure coordinates in such a way that potentially four potassium ions can be bound in the filter *(Fig. 3B).* In addition all Kv types have lysines or arginines as every third or fourth amino acid in the fourth transmembrane helix which are the primary voltage sensor [36, 37, 41].

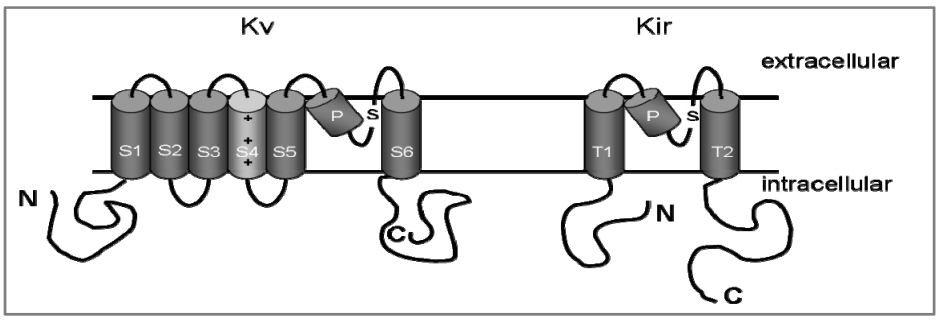


Figure 2: Membrane topologies and main features of the Kv (left) and Kir (right) potassium channel subtypes. Schematic representation of membrane topology of Kv and Kir channels. One subunit of each tetramer is shown for clarity. Transmembrane helices are numbered S1-S6 in Kv channels and T1-T2 in Kir channels. P = pore helix, S = selectivity filter, N = N-terminus and C = C-terminus. The extracellular side is towards the top.

Potassium channels are formed as a tetramer [42] of identical or similar subunits arranged in a fourfold symmetry around the hydrophilic pore which forms the conductive channel. Additionally the core of all potassium channels is formed by the selectivity filter which contains the signature sequence, and is held in place by the pore helix. The most C-terminal transmembrane helix forms the lining of the internal cavity, which at the top of the channel is narrowed by the selectivity filter. At the bottom the cavity is closed by the crossing of four transmembrane helices, giving the channel a teepee like shape.

In general potassium channel activity is tightly regulated, both by tissue specific transcription and by biochemical actions on the channels themselves. However the main task of these channels is selective but high throughput potassium conductance. This is obtained by two main features. First, precise coordination of the dehydrated potassium by the protein; second, multiple ion occupancy sites within the cavity. To establish this the selectivity filter has to be in a conductive conformation, which is obtained by the binding of potassium. However, there are numerous additional features that are suggested to be involved in potassium channel gating and these seem to be (slightly) different for each channel [43-46]. Consequently the exact mechanism of potassium channel gating is still poorly understood.

Direct structural information on potassium channels has sofar mainly been obtained from the crystal structure of KcsA, a channel of the Kir type which is found in *Streptomyces lividans* [31]. This crystal structure, representing the open conformation, was resolved by deleting a large part of the intracellular C-terminal fragment and binding an antibody Fab fragment to the extracellular loops. Recently the crystal structure of the full length closed conformation also in the presence of Fab chaperones was published [47] *(Fig. 3A).* In neither of the crystal structures the N-terminus could be visualized. However EPR studies established that the N-terminus forms a amphiphatic helix that resides on the membrane interface [48].

KcsA will be used as a model protein in the research described in this thesis. However the results we obtain for this channel are rather safe to extrapolate to the entire potassium channel family since the structural assumptions made based on electrophysiological experiments of most eukaryotic potassium channels are in striking harmony with that of KcsA.

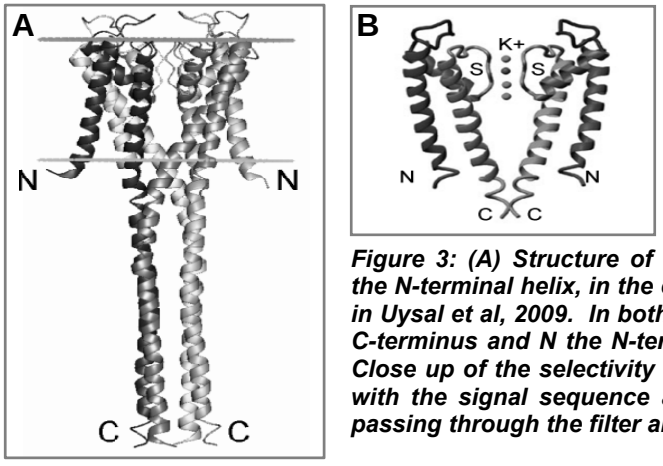


Figure 3: (A) Structure of full length KcsA, lacking the N-terminal helix, in the closed state as described in Uysal et al, 2009. In both figures C represents the C-terminus and N the N-terminus of the protein. (B) Close up of the selectivity filter. S = selectivity filter with the signal sequence and four potassium ions passing through the filter are indicated with K^+.

KcsA

KcsA was first discovered in 1995 under the name SKC1 [49]. Soon it became clear that this small potassium channel was relatively easy to express and purify [50, 51], making it ideal for numerous studies [52-56]. Not long thereafter the crystal structure of the open conformation of the transmembrane part of KcsA was solved [57], giving a lot of insight into the molecular architecture of this channel. KcsA is a symmetric tetrameric channel with each monomer consisting of an N-terminal helix at the membrane interface, a transmembrane helix, followed by a short pore helix leading to the selectivity filter in the tetrameric structure, then a second transmembrane helix and a large C-terminal domain in the cytoplasm *(Fig. 3 and 4).*The structure of the N- and C-terminus were first based on EPR studies [48], of which the C-terminal structure was recently confirmed by X-ray crystallography studies of the closed channel [47]. Binding of the monoclonal antibody Fab fragment to the KcsA tetramer as well as deletion of (part of) the C-terminus are commonly used tools to decrease protein flexibility for these crystallographic studies [31]. The main differences between the open and closed structure of KcsA lies within the region on the interface of the inner membrane, also known as the hinge region [47]. However it is difficult to assess exact differences between the two structures since in both structures the N-terminus is not visualized. Additionally in the full length closed structure the presence of the C-terminus with bound Fab fragments may cause a conformational change in the rest of the structure without it being correlated to gating.

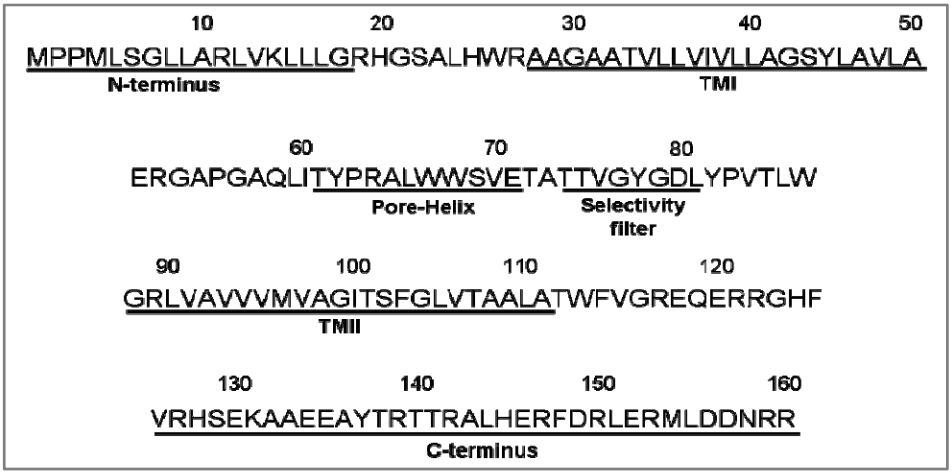


Figure 5: Amino acid sequence of KcsA-WT indicating distinct parts of the protein. The most N-terminal transmembrane helix is indicated with TMI and the most C-terminal transmembrane helix with TMII.

As mentioned before KcsA is often used as a model protein to study ion channel function, e.g. opening probabilities, pH dependence and cluster formation [34, 58, 59]. Additionally also for membrane protein insertion, assembly and stability KcsA is a popular model [33, 60-68]. Assembly and stability are commonly monitored as function of the presence of the small

alcohol trifluoroethanol (TFE) [61, 69]. Moreover KcsA is regularly used as target molecule to investigate binding of toxins and inhibitory molecules which potentially can bind to a wide variety of potassium channels [70-73]. Examples of such inhibitory molecules are the Shaker B ball-peptide which binds to the intracellular part of KcsA and the peptidyl inhibitors and tetraethylammonium which bind to the extracellular pore.

Most relevant for this thesis is that, being an integral membrane protein, KcsA is surrounded by lipids. It has been shown that KcsA can adapt to membrane thickness quite efficiently, probably by tilting its helices [8]. Additionally, tightly bound lipids are present in the crystal structure, suggesting their importance [31]. Moreover it was established that KcsA is influenced by (specifically bound) lipids in assembly, stability [61, 65, 74, 75] and even function [31, 76]. These processes in general and the influence of lipids on these processes specifically will be discussed in the following paragraphs.

Assembly

Assembly of the KcsA monomers into a tetramer has been demonstrated to occur only in the presence of membranes and it was found to be enhanced by the proton motive force [60]. Additionally by making use of an *E.coli in vitro* transcription-translation system it was found that a functional signal recognition particle pathway is essential for tetramer formation, probably to direct correct monomer insertion in the membrane. Strikingly however, the highest efficiency of tetramerization was observed when transcription-translation was carried out in the presence of pure lipid vesicles, demonstrating that a phospholipid bilayer is the minimal membrane requirement to form the KcsA tetramer [33].

Furthermore it was found that properties of lipids as well as of the protein are important for efficient assembly. Membranes containing anionic lipids are found to be most optimal for KcsA membrane association and tetramerization [61]. Furthermore the presence of anionic lipids (PG, PE) is found to allow the transition from unfolded monomer back to the folded tetramer conformation after TFE treatment, whereas in detergent micelles this is only partly the case. This indicates that anionic lipids play a role in the different processes of *in vitro* folding, tetramerization and clustering of KcsA [69].

With respect to features of the protein that are important for assembly, it was found that shortening the C-terminal domain of KcsA beyond residue 124 severely inhibits tetrameric assembly [66]. However, even in the absence of this domain, reconstitution into membrane lipids promotes *in vitro* KcsA tetramerization very efficiently, an event which is likely mediated by allowing proper hydrophobic interactions involving intramembrane protein domains [66]. Moreover this suggests that lipids may act as effectors in KcsA tetramerization.

Stability

Also for tetramer stability it was found that properties of the protein as well as those of lipids are important. The KcsA tetramer is extremely stable, even in a detergent like sodium dodecyl sulfate (SDS) [50], but can be dissociated by heat treatment, high pH [77] or by incubation with small alcohols [31, 78]. The effect of these small alcohols is speculated to be mediated through direct interactions with the protein [79] or indirect via insertion into the lipid

bilayer and affecting properties of the lipid bilayer [74]. However the presence of lipids enhances tetramer resistance against heat [61] and TFE [10]. It has been found that PE stabilizes the KcsA tetramer most likely via membrane lateral pressure [74], whereas PA seems to have a more specific stabilizing effect [75].

With respect to properties of the protein, it was found that the C-terminal domain and the selectivity filter account for some of KcsAs tetramer stability [47, 48, 80, 81]. Especially the presence of potassium bound inside the central cavity, the external vestibule and the selectivity filter promote tetramer stability [67, 68, 80]. Mutations made in (close proximity of) the selectivity filter also indicate that this region is involved in tetramer stability [58, 82]. Moreover two specific mutations in the selectivity filter, G77A and Y78F caused formation of tetramers with reduced stability [82].

For the KcsA C-terminus it is known that especially the formation of a tight four-helix bundle governs tetramer stability [48, 81] and can even be substituted with a non-native tetrameric motif [64]. However, once the KcsA tetramer is assembled, its remarkable *in vitro* stability to alcohol or to heat-induced dissociation into subunits is not greatly influenced by whether the entire C-terminal domain continues being part of the protein [66]. Additionally it is found that the presence of the C-terminus weakens tetramer stability at acidic pH whereas at basic pH values it enhances tetramer stability, suggesting a conformational change which could facilitate gating [83].

In the research described in this thesis we will use the destabilization effect of small alcohols on the KcsA tetramer as a tool to investigate what governs the unique tetramer stability of KcsA. Thereby we focus on regions within KcsA that could be involved in tetramer stability as well as on the role the lipids most likely play in this process.

Gating

Although the channel properties of KcsA have been investigated extensively, the exact mechanism of gating remains unclear. However there is a general consensus on the fact that KcsA most likely has two gates: one at the selectivity filter and one close to the C-terminal domain *(Fig. 5).* The gate at the selectivity filter has shown a necessity for bound potassium to remain in the open state [84, 85]. Additionally is was shown that this gate is voltage dependent and it was suggested to have a C-type inactivation [85, 86]. C-type inactivation of potassium channels involves closing of the extracellular mouth (selectivity filter) of the channels, in contrast to N-terminal mediated (N-type) inactivation in which the intracellular N-terminal domain is involved in closure of the channels. This means that in C-type inactivation shortly after the activation of the potassium channel, the selectivity filter stops conducting ions depending on various stimuli. The molecular mechanism of C-type inactivation in KcsA is not fully understood yet, but it has been found that mutations in or near the selectivity filter give rise to an increased open probability [87-89] indicating that this region is involved in the C-type inactivation.

Also for gating specific properties of lipids and of the protein are of importance. Tightly bound lipids, that were identified as PG, were found in the KcsA crystal structure indicating a specific role [31]. These lipids bind to a cleft between two adjacent monomers, which was found to be a general high affinity binding site for lipids, with PA>PG>>PE in terms of preferential protein-lipid

interactions [76, 90]. Furthermore a KcsA mutant was made in which W67 and W68, close to the lipid binding pocket, remained unaltered but W27, W87 and W113 were replaced by a leucine facilitating local tryptophan fluorescent measurements. By making use of this mutant in fluorescence quenching studies it has been sown that binding of anionic lipids to at least three of these lipid binding sites is essential for gating [91]. This implies that possibly the binding of (specific) lipids to this cleft might play a role in the (in)activation of the selectivity filter.

KcsA is found to open at low pH [92, 93]. The pH sensor is most likely formed by E118, E120 and H25 which lay close to the C-terminal domain. This is supported by the observation that mutating these residues into alanines results in a constitutive open channel till a pH of 9.0 [89, 94]. Additionally W67 is suggested to be involved in pH sensing based on its pH dependent chemical shift in NMR experiments [93]. However others claim that the C-terminal domain itself is the pH sensor since it destabilizes tetramer stability at acidic pH whereas at basic pH tetramers stability was enhanced [83]. Additionally it was found that in the chimeric protein KcsA-Kv1.3 as well as in KcsA-WT these two gates are most likely coupled, resulting in coordinated sequential opening [93, 95-97]. However the exact mechanism of this coupling remains elusive.

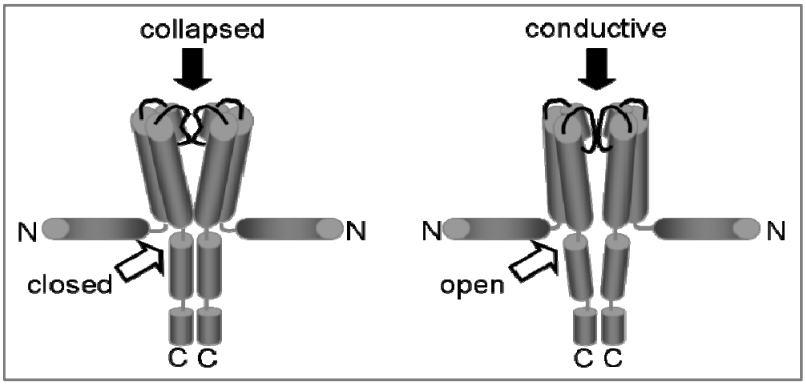


Figure 5: Cartoon schematically depicting the two separate gates within KcsA. The selectivity filter is indicated with a black arrow, and the gate at the inner membrane interface with a white arrow. C represents the C-terminus and N the N-terminus of the protein. Both gates are depicted in the closed (left) and open (right) conformation. Only two monomers are depicted for clarity.

At present, various opinions circulate about the general conductive properties of KcsA. For KcsA-WT several different conductive states with an open probability of 0.5 and triggered by low pH, have been found by the group of Schrempf [35, 82, 98]. In contrast Millers group only finds one conductive state with a much lower open probability [99, 100]. Finally the group of Gozalez-Ros claims that KcsA has a diverse functional behavior, in which the most frequently observed pattern of activity corresponds to the low open probability and acidic pH-dependent state, but additional states were also observed. These additional states are characterized by a high open probability at acidic as well as neutral pH and show frequent coupled gating of multiple channels [34].

Together the findings above indicate that KcsA has two separate gates that might be coupled in gating. Furthermore it seems that KcsA can have several conductive states, which are most likely regulated via these gates by low pH, the presence of potassium and possibly the binding of lipids. Additionally KcsA tetramers are most likely able to form clusters in which coupled gating can be initiated [34, 101].

In this thesis, besides looking at the influence of lipids and protein properties on tetramer stability we also explore if lipid binding to the KcsA tetramer, plays a role in the functional behavior.

Scope of this thesis

KcsA is a well studied membrane protein, but not all characteristics are well understood. The aim of this thesis is to obtain more insight in the influence that lipids have on KcsAs tetramer stability and gating behavior. In chapter two we identified regions within KcsA that can enhance tetramer stability. This was done by making drastic replacement and deletion mutations within defined KcsA regions. Subsequently the tetramer stability of these mutants was studied by TFE titration and visualized by a gel-shift assay using SDS-PAGE.

The influence of lipids on this tetramer stability was further explored in chapter 3. Especially the molecular mechanism behind the stabilizing effect of PA on the KcsA-WT tetramer is further investigated. Interestingly we found that the chimeric potassium channel KcsA-Kv1.3 is not stabilized by PA, likely due to changes in protein charge on the interface between monomers. This implies that the lipid binding site lies within the mutated region of this channel.

In chapter 4 we investigate whether the R64D mutation in KcsA-Kv1.3 is responsible for the loss of stabilization by PA. This was studied by introducing a R64D or R64A mutation in KcsA-WT and subsequently visualizing tetramer stability with SDS-PAGE upon TFE titration. Indeed specificity for PA was lost in both KcsA-R64A and KcsA-R64D, but surprisingly, KcsA-R64D had an exceptionally high tetramer stability compared to KcsA-WT. This enhanced stability is probably obtained by the formation of a salt bridge between R89 and D64 of adjacent monomers. Additionally mass spectrometry experiments on native KcsA-WT show that the tetramer dissociates into two dimers, rather than a trimer and monomer, implying an allosteric mechanism for lipid binding.

In chapter 5 we found that the gating properties of KcsA-R64A and KcsA-R64D are similar to those of KcsA-WT. However the mutant channels appear to be less stable compared to KcsA-WT which results in a higher deactivation percentage in these mutants. This suggests that the increase in tetramer stability of KcsA-R64D observed in chapter 3 correlates to the irreversible closed state observed in chapter 5. It is proposed that this is due to a stable interaction that mimics lipid binding. Chapter 6 discusses the results obtained in this thesis in relation to literature and highlights some future perspectives.

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Chapter 2

KcsA tetramer stability is based on multiple protein characteristics

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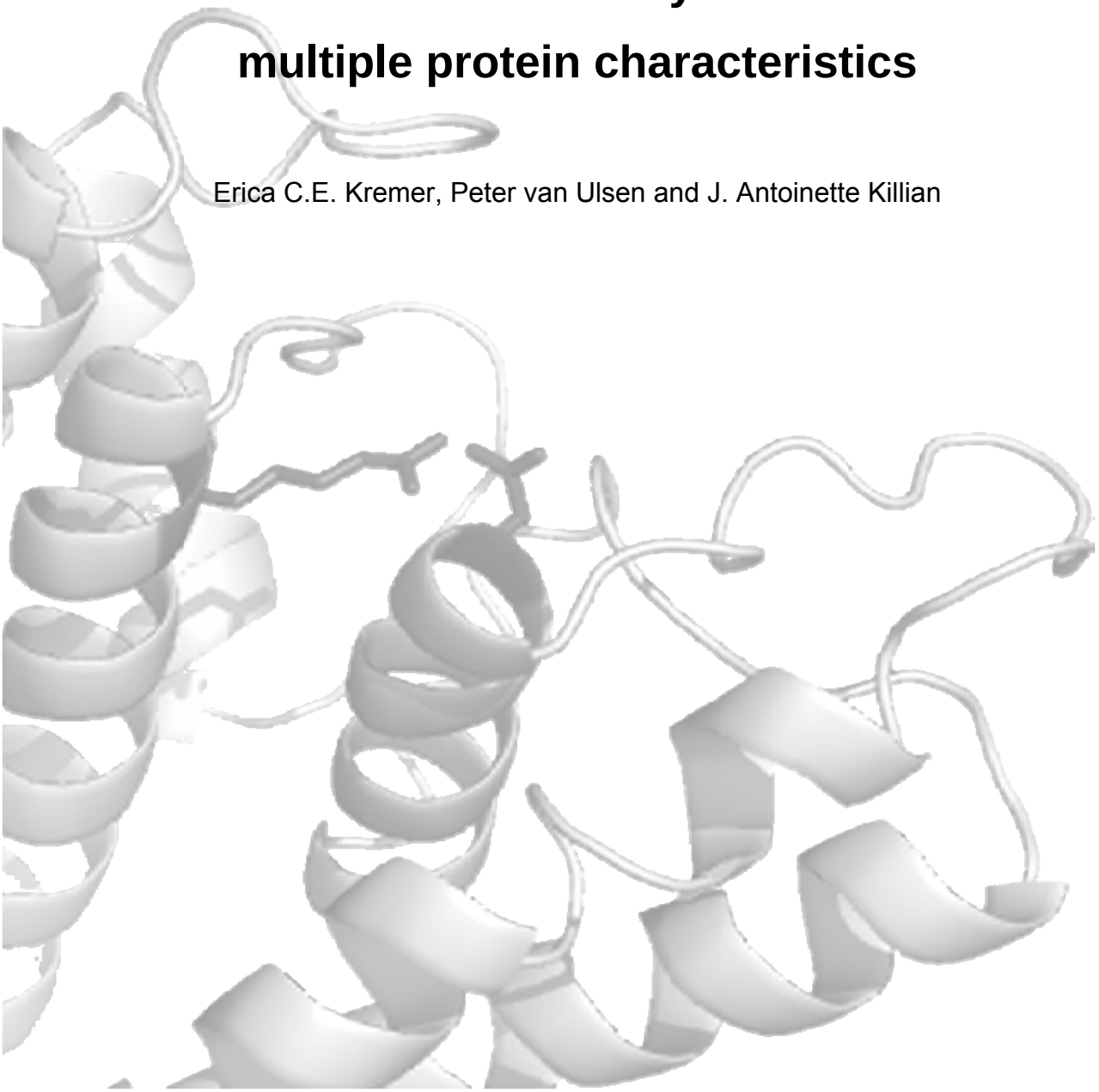


Abstract

The potassium channel KcsA forms a stable homotetramer which can be detected with SDS-PAGE. It had been shown that the C-terminal part and the selectivity filter of KcsA contribute to its stability. In this study we focused on the role of the transmembrane helices and the pore region in the formation of and the stability of the tetramers. We first introduced single amino acid substitutions in the transmembrane regions and analyzed their effect using trifluoroethanol (TFE) stability assays. We found no significant effects of these mutations on tetramer stability, either *in vivo* in the *E.coli* membrane or when purified in detergent micelles. In a rather bold approach, we subsequently replaced entire transmembrane segments and deleted defined parts of the pore region of KcsA. The expression of these replacement and deletion mutants was low and poorly reproducible, prohibiting a detailed analysis of KcsA stability. Nevertheless, SDS-PAGE experiments clearly showed that neither the amino acid composition of the helices nor the presence of the pore region is essential for tetramer formation. Our results suggest that both the C-terminus as well as the pore region contribute to KcsA tetramer formation and stabilization.

Introduction

Approximately 25% of all proteins encoded in genomes are transmembrane or membrane-associated proteins [1], hence this category of proteins is of great importance for life. For many classes of membrane proteins, such as translocators and channels, it is known that they may function as part of oligomeric complexes [2]. These complexes can consist of multiple subunits of the same or different proteins [3, 4]. One of these oligomeric proteins is the potassium channel KcsA from *Streptomyces lividans (Fig. 1).* KcsA is well-studied [5-7], mainly due to the fact that a crystal structure of the transmembrane part is available [8]. It is a homotetrameric protein that exhibits extreme tetramer stability. Even when KcsA containing membranes are subjected to temperatures up to 90°C in SDS the protein remains in its tetrameric form. Previously it was found that the KcsA tetramer can be destabilized by small alcohols [9] and dissociate into monomers depending on the concentration and type of alcohol. Therefore, monitoring tetramer dissociation as a function of alcohol concentration with SDS-PAGE is a convenient tool to investigate KcsA tetramer stability in more detail [10, 11].

In this study we prepared mutant KcsA proteins to investigate what governs the exceptional tetramer stability of KcsA. Each KcsA monomer consists of an N-terminal helix at the membrane interface, a transmembrane helix, followed by a short pore helix leading to the selectivity filter in the tetrameric structure, then a second transmembrane helix and a large C-terminal domain in the cytoplasm. It has already been established that the presence of the C-terminus and the selectivity filter account for some of the stability of this protein [5, 12-14]. Since it is known that the lipid environment plays a role in the stability of KcsA [15], it seems reasonable to assume that also the transmembrane parts (TM) of the protein will be involved. To test this we introduced specific mutations into this part of KcsA. More specifically we

introduced single amino acid substitutions in TMI, we replaced TMI and TMII by an alanine/leucine stretch and mutants with significant deletions in the pore region of the protein were made. It was found that the *in vivo* stability of the point mutants was representative for the stability of the purified protein in DDM. For the mutants with altered transmembrane segments or pore region deletions, tetramers could be observed in some experiments, suggesting that the pore mutants have a different shape compared to KcsA-WT, and that in these mutants the C-terminus is the dominant tetramerization motif.

Materials and methods

Materials

The following chemical reagents were purchased either from Fluka, Merck or Sigma Aldrich, available in the highest purity: Lysozyme, HEPES, KCl, NaCl, imidazole and isopropyl-β-D-thiogalactopyranoside (IPTG). n-Dodecyl-β-D-maltoside (DDM) was obtained from Anatrace Inc, Ni^{2+} - NTA agarose from Qiagen, trifluoroethanol (TFE) from Merck and Coomassie Brilliant Blue G-250 from ICN Biomedicals. The electrophoresis setup and markers were purchased at Biorad Laboratories B.V. Ultra-pure agarose was obtained from Invitrogen, Taq polymerase from Fermentas, the Quickchange site directed mutagenesis kit from Stratagene and the monoclonal anti-polyhistidine-peroxidase from Sigma Aldrich.

KcsA mutagenesis

Point mutations in *kcsA* were introduced using the Quickchange mutagenesis kit according to the recommendations by the manufacturer. (Primer design and PCR were optimized by following the supplied protocol.) Pt7-837KcsA-WT was used as template DNA. Obtained mutations were verified by DNA sequencing. KcsA-TM replacement and KcsA pore deletion mutants were obtained by a customized PCR-based method (see appendix).

Protein expression and purification

KcsA was expressed and purified, with some minor adaptations, as described previously in [15]. Briefly, KcsA-WT (or mutant) was expressed with an N-terminal His-tag from pT7-KcsA in *Escherichia coli strain BL21 (DE3)* upon IPTG induction. After collection cells were lyzed in a French press to obtain membrane fractions for *in vivo* studies. Alternatively the proteins were purified in a buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM KCl. The solubilized membranes in 3 mM DDM were incubated with pre-washed Ni^{2+}-NTA agarose beads overnight at 4 °C. The bound His-tagged proteins were eluted with the HEPES buffer described above containing 300 mM imidazole and 1 mM DDM. The purity of proteins was assessed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration and purity was assessed by SDS gel after staining with Coomassie Blue, using a standard of bovine serum albumin (BSA).

SDS-PAGE stability assay

This assay was performed as described in [16]. Briefly, membrane fractions of cells overexpressing KcsA, or purified protein in DDM were divided in equal aliquots of 10 µl containing approximately 2,5 µg of protein and incubated with increasing amounts of TFE for one hour at room temperature. TFE was added in an aqueous solution with a total concentration of 5 mM KCl, 45 mM NaCl to minimize changes in ionic strength. The TFE containing samples, with a total volume of 13 µl, were mixed with 5 µl of an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8,50% glycerol, 0.01% bromophenol blue and 10% SDS) and directly run on 15% acrylamide gel in the presence of 0.1% SDS at room temperature. Afterwards proteins were visualized either by Western blot in combination with an α-His antibody for the *in vivo* samples or with Coomassie Brilliant Blue staining for the purified proteins. The gels containing purified protein samples were scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The tetramer band intensity present at 0 vol% TFE was set to 100%. The amount of tetramer (%) was plotted against the TFE concentration (vol%) for the stability assay.

Tryptophan fluorescence

All fluorescence experiments were performed as described in [16]. Briefly, samples in a purification buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM KCl at room temperature using a Varian Cary Eclipse spectrofluorometer in a 200 µl quartz cuvette. The samples were excited at 280 nm and emission spectra were collected between 300 and 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm. Spectra were corrected for imidazole contribution afterwards.

Results

Specific mutations

It had been shown that protein-lipid interactions play a role in KcsA tetramer stability [15, 16]. Therefore, it is possible that residues in the outer helix of KcsA, facing the lipid bilayer, play a role in this stabilization. To identify such residues, single amino acid substitutions were introduced in the outer helix (TMI) of KcsA *(Fig 1A).* In particular, we substituted residues G43 and Y45 for an alanine to investigate whether altering these amino acids could affect tetramer stability. G43 and Y45 are located on the top half of the outer helix mostly facing the lipid bilayer *(Fig. 1B)*.

To determine whether these mutations influence tetramer stability the mutant proteins were expressed in *E.coli*. After harvesting, stability was tested on membrane fractions using heat or TFE incubation and assessed by Western-blot analysis using an antibody against the His-tag. *Fig. 2A* shows typical results of the comparison of the stability of the mutants with KcsA-WT upon heat incubation. A clear tetramer band is visible for KcsA-WT at 64 kDa, which dissociates into a monomeric band of around 18 kDa under exposure to temperatures exceeding 90°C. The KcsA-G43A behaved similarly and completely dissociated between 90°C and 99°C, while for mutant KcsA-Y45A a tetrameric complex is clearly detectable after 99°C incubation. However, at

these transition stages the differences in the percentage tetramer present was considerable in similar experiments, thereby generating a substantial error. If the average percentage of tetramer of several experiments is plotted against temperature no significant difference between the two KcsA-mutants compared to KcsA-WT is found *(Fig. 2C)*. *Fig. 2B* also shows that after dissociation by TFE both mutants appear to remain dully complexed in a tetrameric complex, whereas KcsA-WT only partially remains complexed. However, and similar as observed for the temperature incubations, in the TFE experiments the error is relatively large and a comparable stability for both mutant proteins compared to KcsA-WT is found if the average percentage of tetramer of several experiments are plotted against the vol% TFE present *(Fig. 2D).* Furthermore it should be noted that in the TFE stability experiment represented in *Fig. 2B* no monomeric band could be observed for either the mutants or the WT protein. Probably this is due to the fact that TFE shields the monomer from antibody detection [10].

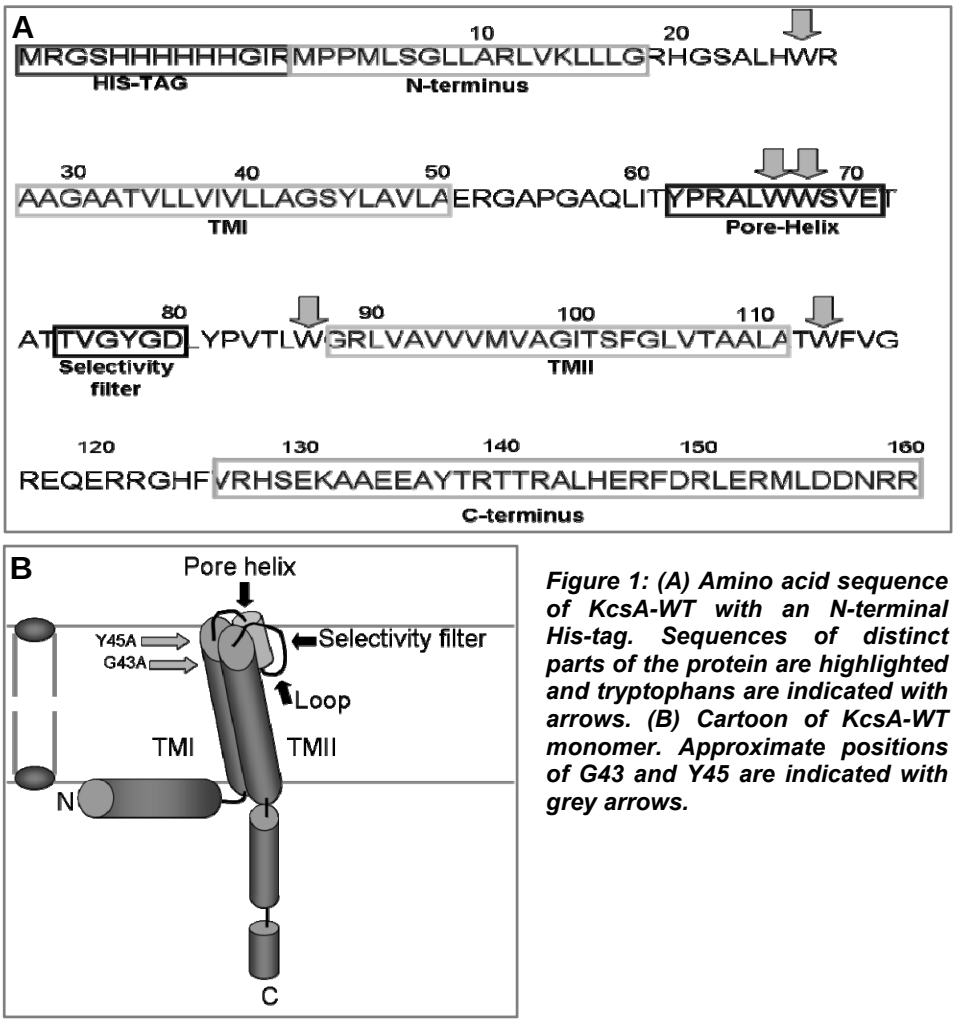


Figure 1: (A) Amino acid sequence of KcsA-WT with an N-terminal His-tag. Sequences of distinct parts of the protein are highlighted and tryptophans are indicated with arrows. (B) Cartoon of KcsA-WT monomer. Approximate positions of G43 and Y45 are indicated with grey arrows.

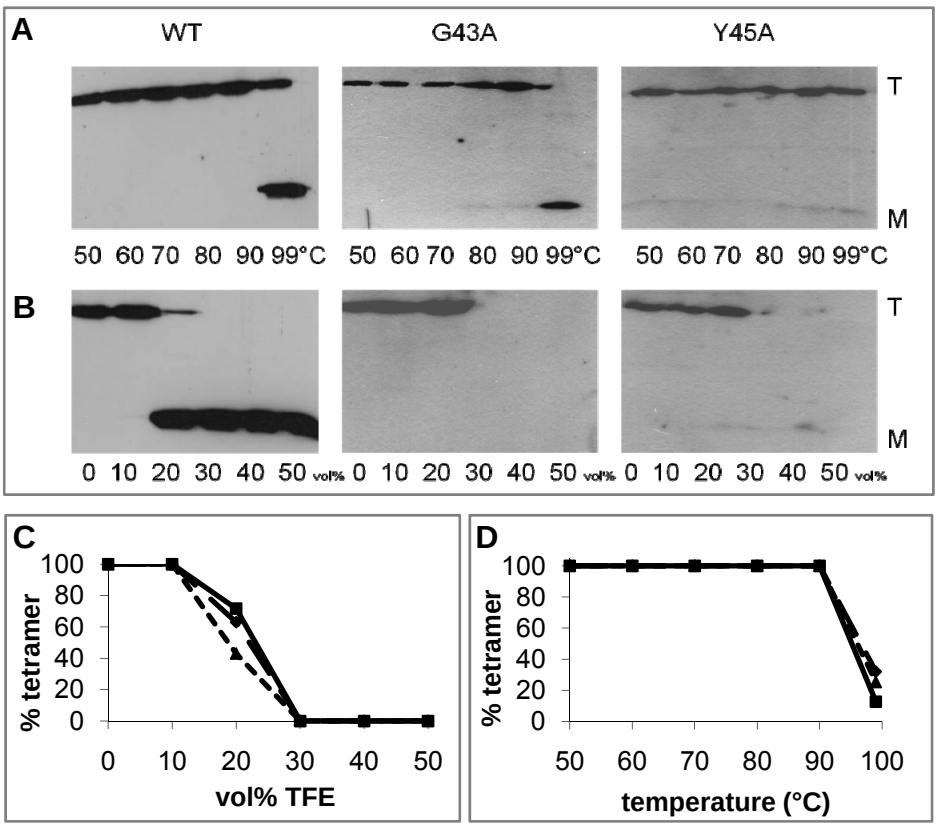


Figure 2: In vivo tetramer stability assay. Representative Western blots are shown of KcsA tetramers (T) and monomers (M) upon heat (A) or TFE titration (B). Proteins were detected with αHis-antibody. Quantification of tetramer bands on blot is plotted as percentage tetramer as a function of heat (C) or TFE (D). Solid line (■) represents KcsA-WT, dashed line (♦) KcsA-G43A and dotted line (▲) KcsA-Y45A. Data points show the average of at least three experiments. For most points an error less than 5% was estimated. However for points around the transition from tetramer to monomer a large deviation was found. The maximal error was estimated to be 30% at 99°C and 40% at 20 vol % TFE.

In the *in vivo* assay it is difficult to determine whether the observed tetramer stability is really the same in all three proteins or whether additional factors might play a role, such as for example small variations in lipid composition, the presence of other proteins, or small differences in ion composition and ionic strength. To exclude these factors, KcsA-WT and mutants were purified in DDM and TFE dissociation studies were performed. *Fig. 3* shows that also in DDM both mutants show similar stability compared to KcsA-WT. The error at the transition stage between experiments in DDM was smaller (max 10%) compared to the *in vivo* situation (30-40%). This implies that, although a significant error was found at the dissociation point *in vivo*, the obtained tetramer stability *in vivo* fairly well reflects the stability in the isolated proteins.

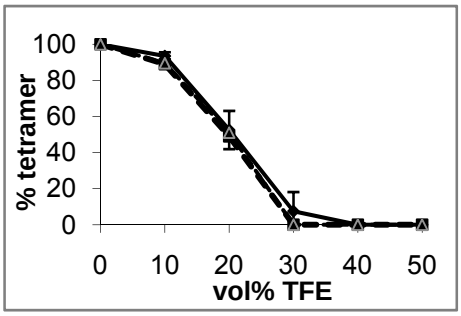
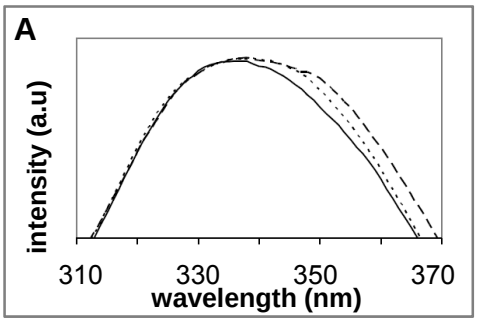
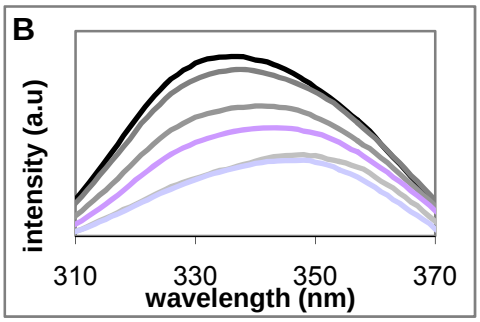


Figure 3: TFE stability in DDM. Percentage of tetramer is plotted as function of the concentration of TFE present. Solid line (■) represent KcsA-WT, dashed line (♦) KcsA-G43A and dotted line (▲) KcsA-Y45A. All experiments, at least three for each data point, were performed in a buffer containing 5 mM KCl and at pH 7.4. The maximal error was 10% for KcsA-WT at 20 vol% TFE.

Since results obtained from an SDS-gel assay might be influenced by the presence of SDS in the gel, tryptophan fluorescence was used to verify these results *(Fig. 4)*. In DDM we find a maximal fluorescence intensity for KcsA-WT at 337 nm. KcsA-Y45A and even more so KcsA-G43A show a small red shift compared to KcsA-WT, indicating that in these two mutants the tryptophans might be in a slightly more hydrophilic environment compared to KcsA-WT.





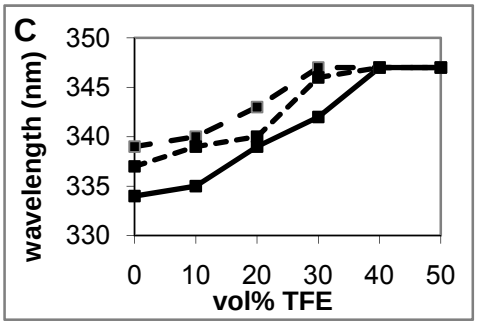


Figure 4: Results of tryptophan fluorescence experiments for KcsA-WT (solid line) KcsA-G43A (dashed line) and KcsA-Y45A (dotted line) in DDM. (A) Fluorescence emission spectra without TFE present. (B) TFE titration from 0-50 vol% on KcsA-WT, in steps of 10 vol%, with increasing concentration of TFE being represented with decreasing color intensity of the lines. (C) Wavelength of maximum as function of vol% TFE. All experiments were performed in a buffer containing 5 mM KCl and at pH 7.4.

Transmembrane helices

From the results obtained from the single amino acid substitution mutants we can conclude that the residues G43 and Y45 in the outer helix are not involved in tetramer stability, even though small structural rearrangements seem to occur. However in general it can be assumed that the overall composition of the outer helix (TMI) influences the protein-lipid interface as well as the helix-helix

interactions within KcsA. This would suggest that tetramer formation and stability could be compromised when the composition is significantly altered. To investigate whether this is indeed the case, a fairly drastic strategy was taken to replace TMI entirely by an alanine/leucine stretch. The cloning strategy for this mutant is described in the appendix. After harvesting the *E.coli* cells overexpressing the proteins, membrane fractions were obtained and proteins were detected with SDS-PAGE by an α-His antibody. It was found that expression of this mutant was very irregular, indicating that expression rates of these proteins differed severely in similar experiments. Additionally also the multimeric form of the protein varied per experiment, Besides tetramers and monomers, commonly also dimers, trimers, monomers and sometimes even aggregates were present. Remarkably however in some experiments at 64 kDa a KcsA-TMI tetramer could be detected on gel *(Fig. 5 left lane),* demonstrating that despite alteration of the helix composition, formation of a tetramer, stable enough to withstand dissociation by SDS, is still possible. The irreproducible expression patterns, unfortunately, made it impossible to perform stability assays as described for KcsA-G43A and KcsA-Y45A.

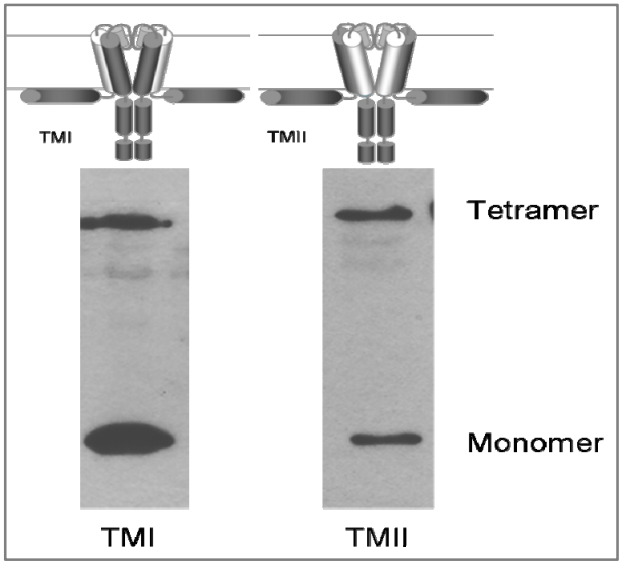


Figure 5: Western blots of in vivo expression of KcsA-TMI and KcsA-TMII showing tetramer and monomer bands. Proteins were visualized with an α-His-antibody. Above the blots a cartoon of KcsA-TMI and KcsA-TMII are shown with the mutated helix depicted in white. Only two monomers a depicted for clarity.

Since it seems to be possible to replace the outer helix with an alanine/leucine stretch we used the same approach to investigate the influence of the composition of the inner helix on tetramer formation. Membrane fractions of KcsA-TMII were obtained and assayed as described for KcsA-TMI. Again expression was irreproducible and similar KcsA forms as described for KcsA-TMI were observed regularly. However in some experiments also KcsA-TMII was found to be still able to form tetramers with sufficient stability to remain tetrameric in SDS-PAGE *(Fig. 5 right lane).* This indicates that also the composition of the inner helix is not of crucial importance for tetramer stability. Also here the irreproducibility of the expression prohibited further characterization of the stability.

Pore region

Tetramer stability is influenced by cations, *e.g.* Potassium, that bind inside the central cavity, the external vestibule and the selectivity filter [13, 17, 18]. Mutations in (close proximity of) the selectivity filter also influenced tetramer stability [19, 20]. Next, we analyzed whether the presence of the selectivity filter, with its bound cations, or the complete pore region is essential for tetramer formation. Several deletion mutants were constructed that lacked a specific part of the pore region *(Fig. 6A):* ΔS has no selectivity filter, ΔPS has a deletion of the pore helix and selectivity filter, and ΔPSL lacks the loop (TAT) between the pore helix and the selectivity filter as well. For details of specific amino acids within these deletions see *Fig. 1A*. Since the pore region forms the core of the characteristic teepee shape of the KcsA tetramer, it is unlikely that these deletion mutants will still be able to assemble in a similar way compared to KcsA-WT.

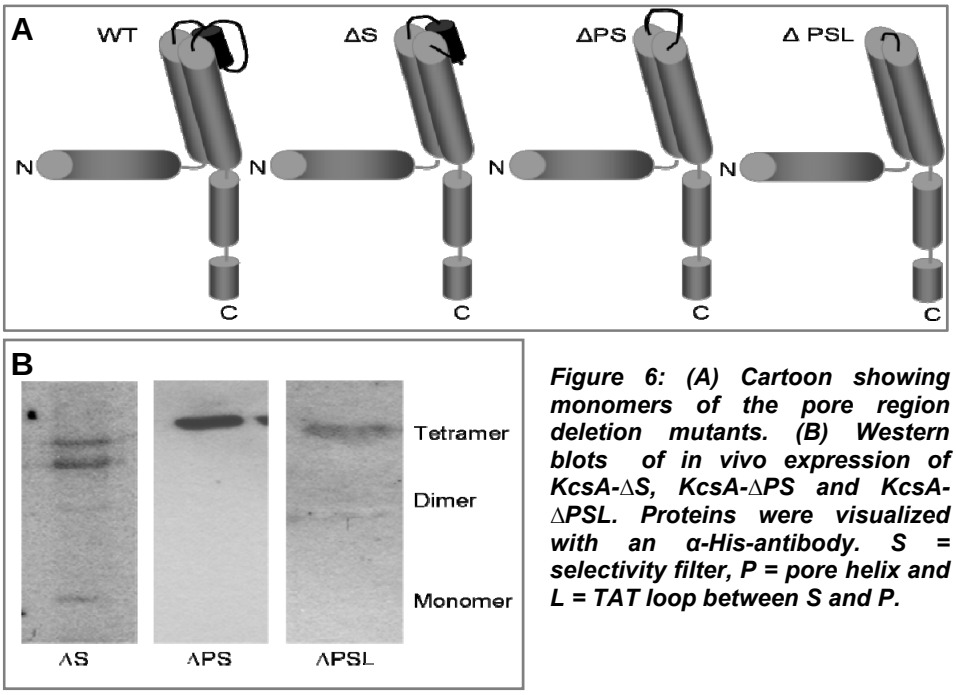


Figure 6: (A) Cartoon showing monomers of the pore region deletion mutants. (B) Western blots of in vivo expression of KcsA-ΔS, KcsA-ΔPS and KcsA-ΔPSL. Proteins were visualized with an α-His-antibody. S = selectivity filter, P = pore helix and L = TAT loop between S and P.

First the oligomerization state of the expressed protein was checked as described for the TM mutants. Expression patterns, again, were irreproducible and prevented further stability studies. However, and against expectations, occasionally tetramers could be observed for all three mutants, as illustrated in *Fig. 6B.* For KcsA-ΔS a tetramer doublet that runs a bit lower compared to KcsA-WT is observed, which probably reflects conformational rearrangements within the protein that will be discussed later. Furthermore, it should be noted that in the expression studies of these mutants often other protein bands were present that may represent dimers, trimers or tetramers of the complex with a different conformation. Together, the results suggest that the pore of the protein by itself is not essential for a tetrameric protein.

Discussion

This study explores what properties govern the remarkable stability of the tetrameric potassium channel KcsA. It is already known that the C-terminus is involved since its deletion leads to less resilience against heat at basic pH [5, 21]. Moreover, it has been reported that KcsA tetramer assembly is affected *in vivo* when the C-terminus is shortened [22]. Besides the C-terminus also the pore region has been put forward as being involved in tetramer stabilization [20]. Especially the binding of various inorganic cations to this region is known to enhance tetramer stability [13, 17]. In this study we addressed the possible involvement of the trans-membrane helices and the entire pore region in tetramer stability.

The basis of this study lies in introducing mutations in the protein that vary from minor amino acid substitutions, to more drastic replacements or deletions of regions. By making amino acid substitutions in the outer helix, at the side facing the surrounding lipids, we found that these residues are most likely not involved in tetramer stabilization. This indicates that the composition of the residues forming the lipid interface in the outer helix may not be of importance for tetramer stability. This was supported by the observation that a mutant in which the entire outer helix was replaced by an alanine/leucine stretch, despite the difficulty in expressing the protein, it was still able to form tetramers that were stable enough to withstand SDS-PAGE. Surprisingly, this may indicate that the residue-composition in the protein-protein interface are not of great importance for tetramer stability. In a similar way it was established that the composition of the inner helix of KcsA was not essential for tetramer formation. Together this indicates that the composition of both transmembrane helices can be varied to a large extent before the tetramer dissociates upon SDS-PAGE.

Subsequently, we investigated whether the pore region is essential for tetramer formation and stability, or whether the binding of cations to the selectivity filter adds to the stability already present. We constructed mutants that had significant parts in the pore region deleted. Against all expectations, we detected tetramers for all mutants. This indicates that the presence of the pore region is not essential for tetramer formation. Moreover, it suggests that the binding of cations to the selectivity filter only adds to an intrinsic tetramer stability that is already present. Together this implies that the four helix bundle of the C-terminus is most likely to be the dominant player in tetramer stability, but that the presence of the pore region with cations bound can add to this basis stability. We will now discuss the influence of the various mutations on tetramer stability in more detail.

Specific mutations

Previously it was found in our lab that the KcsA-WT tetramer in DDM can withstand 10-20 vol% TFE before dissociating [10] However here we find that the KcsA-WT tetramer in DDM dissociates between 20-30 vol% TFE. A likely explanation for this small discrepancy is the small variation in potassium that occurs upon adding TFE, since it is known that the KcsA-WT tetramer becomes less stable at potassium concentrations below 5 mM [17]. In previous

studies the loss of ionic strength was not corrected for, which possibly contributed to the observed lower stability of the KcsA tetramer in those studies.

For KcsA-G43A and KcsA-Y45A no altered tetramer stabilization was observed with SDS-PAGE, indicating that these residues are not directly involved in general tetramer stability. Inhomogeneity of the *in vivo* samples is probably to a large extent responsible for the large error at the transition point. This is confirmed by the smaller error that is found at the transition point for the more homogeneous purified samples.

In the fluorescence studies a maximum of 337 nm was found for KcsA-WT whereas in previous studies a maximum intensity for KcsA-WT was found around 324 nm in mixed micelles or vesicles [16, 18, 23]. In the present study imidazole was not removed before the fluorescence measurements, which made it necessary to correct for this additional fluorescence afterward. This correction most likely is responsible for the shift of the maximum compared to literature. Nevertheless, a small but significant shift was observed for both KcsA-G43A and KcsA-Y45A that indicates a slightly more hydrophilic environment for the tryptophans in these mutants. The mutated residues normally are located on the outer helix close to the point where this helix interacts with the pore helix. Apparently, mutating these residues most likely resulted in a conformational change in such a way that the tryptophans on this helix (W67and W68) became more exposed to a hydrophilic environment. The reason that KcsA-G43A shows a larger red shift than KcsA-Y45A is probably due to fact that G43 is located more towards the pore helix whereas Y45 is positioned towards the outside of the protein/lipid environment. Overall, the results indicate that the mutations cause minor conformational changes that do not affect tetramer stability. This may suggest that *in vivo* KcsA obtains its stability from intra-protein interactions, possibly supplemented by tightly bound lipids or co-factors, rather than from interacting with its lipid environment.

Transmembrane helices

There could be several reasons why the expression patterns of the KcsA-TM mutants is so irregular. First it could be that these mutants are more toxic to the cells compared to KcsA-WT, which means that they might end up in the degradation machinery. However, after expression no signs of severe toxicity, such as dying/lysing cells or the formation of inclusion bodies, were observed. Furthermore various mutant protein complexes were observed on gel, that were not seen for KcsA-WT. These most likely reflect complex intermediates, suggesting that these TM mutants have problems in insertion, assembly and/or stability. It has been shown that a functional signal recognition particle pathway is essential for tetramer formation *in vivo* [24]. Since KcsA does not contain a signal sequence, and a KcsA mutant lacking the N-terminal amphipathic helix expresses as a tetramer (chapter 3), it seems likely that the SRP uses TMI as recognition motif. Consequently one can imagine the SRP machinery might have difficulties in properly recognizing the KcsA with an altered helix and thereby causing mislocalization of the monomers. If they do reach the membrane, assembly into tetramers can take place. Since not much is known about KcsA tetramer assembly it is difficult to speculate on how altering helices would affect this process. As for the tetramer stability, it is likely

that altering a transmembrane helix would affect this property, especially since protein-lipid as well as protein-protein interactions rely on the composition of these helices. Taken all these possible reasons for failure into account, it is even more intriguing that sometimes the cell manages to produce, insert and assemble KcsA-TM tetramers that are stable enough to be detected with SDS-PAGE. Because no additional assays were performed, no information on the exact structure or functionality of the protein could be obtained. Therefore the possibility arises that these KcsA-TM mutants have a different structural conformation compared to KcsA-WT, especially since alteration in helix composition is likely to affect the helix-helix interactions. Consequently the transmembrane helices as well as the pore helices could be oriented in a more compact way compared to KcsA-WT thereby causing the protein to lose its characteristic teepee shape, as described for the pore mutants below. Hence at this point we can not state with certainty whether the helix composition of transmembrane helices in KcsA, is or is not involved in tetramer stability of the native protein.

Pore region

Also for the pore deletion mutants troublesome expression was experienced. Since no unnatural sequences were added, for these mutants especially insertion, assembly and stability might play a role in this expressional irreproducibility. The fact that tetramers are still present in these mutants was very unexpected since it has been published that the presence of potassium in the selectivity filter stabilizes the KcsA tetramer [13, 18]. However it could be that the presence of the selectivity filter itself destabilizes the tetramer, whereas the filter with potassium bound to it does have a stabilizing effect. Since in our mutants the selectivity filter is deleted there has to be another way by which tetramer stabilization is obtained. It is known that the C-terminus of KcsA forms a tight four-helix bundle that provides tetramer stability [5, 12]. The formation of this bundle consequently brings the lower part of the inner helices in close proximity. This in combination with the fact that some of the observed tetrameric bands run a little bit lower on an SDS gel than the expected 64 kDa might indicate that these mutants have a more compact conformation compared to KcsA-WT. The lack of the stabilization introduced by the selectivity filter together with the tight association of the tetramer at the lower part of the inner helices might result in an increase in helix/helix interactions. This would imply that in our mutants these helix/helix interactions are not solely present in the C-terminus but also in the transmembrane part. Thus what we observe on gel might be a eight helix bundle which no longer has a teepee shape or a channel in the middle *(Fig. 7).* This model implies that the pore region is not important for general tetramer stability, although of course it is essential for the formation of a potassium selective stable pore through the protein.

Together the findings described above suggest that the C-terminus is the dominant factor for tetramer formation of the transmembrane helices. This would imply that with the pore region deletion mutants we have created a model protein which can assemble by itself into stable oligomeric structures in *E.coli* membranes. By combining these deletion mutants with the KcsA-TM mutants it could open possibilities for systematic studies on factors involved in membrane protein, oligomer stability, oligomerization and protein-lipid interactions in

general. Similar to the WALP system that is used as a model for membrane spanning peptides [25] a model system could be developed, but now for self assembling oligomers. However to accomplish this, first the expressional problems of these mutants must be overcome, allowing to obtain these mutants in a purified form suitable to function as a model system. This will not be a straight forward approach. An intriguing alternative to overcome the problems of expression, membrane insertion and assembly *in vivo* is to use an *in vitro* transcription translation assay in which KcsA-WT is known to express, insert and oligomerize efficiently in the presence of synthetic lipid vesicles. Since the lipid composition can be varied [15], this assay could be used as a tool for stability studies as well as for further investigation into the effects of lipids on, assembly and stability of membrane proteins. Also this will not be an easy assay to set up, but it would provide an elegant tool to systematically investigate the role of specific protein/protein and protein/lipid interactions in structural and functional properties of membranes.

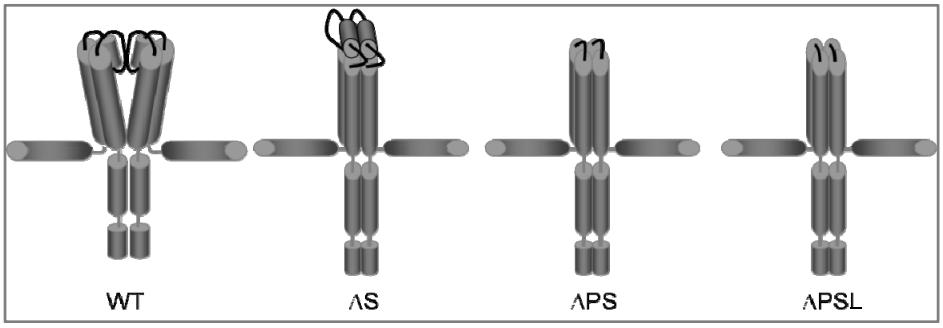


Figure 7: Model for conformation of the pore deletion mutants, dimers are shown for clarity. S = selectivity filter, P = pore helix and L = TAT loop between S and P.

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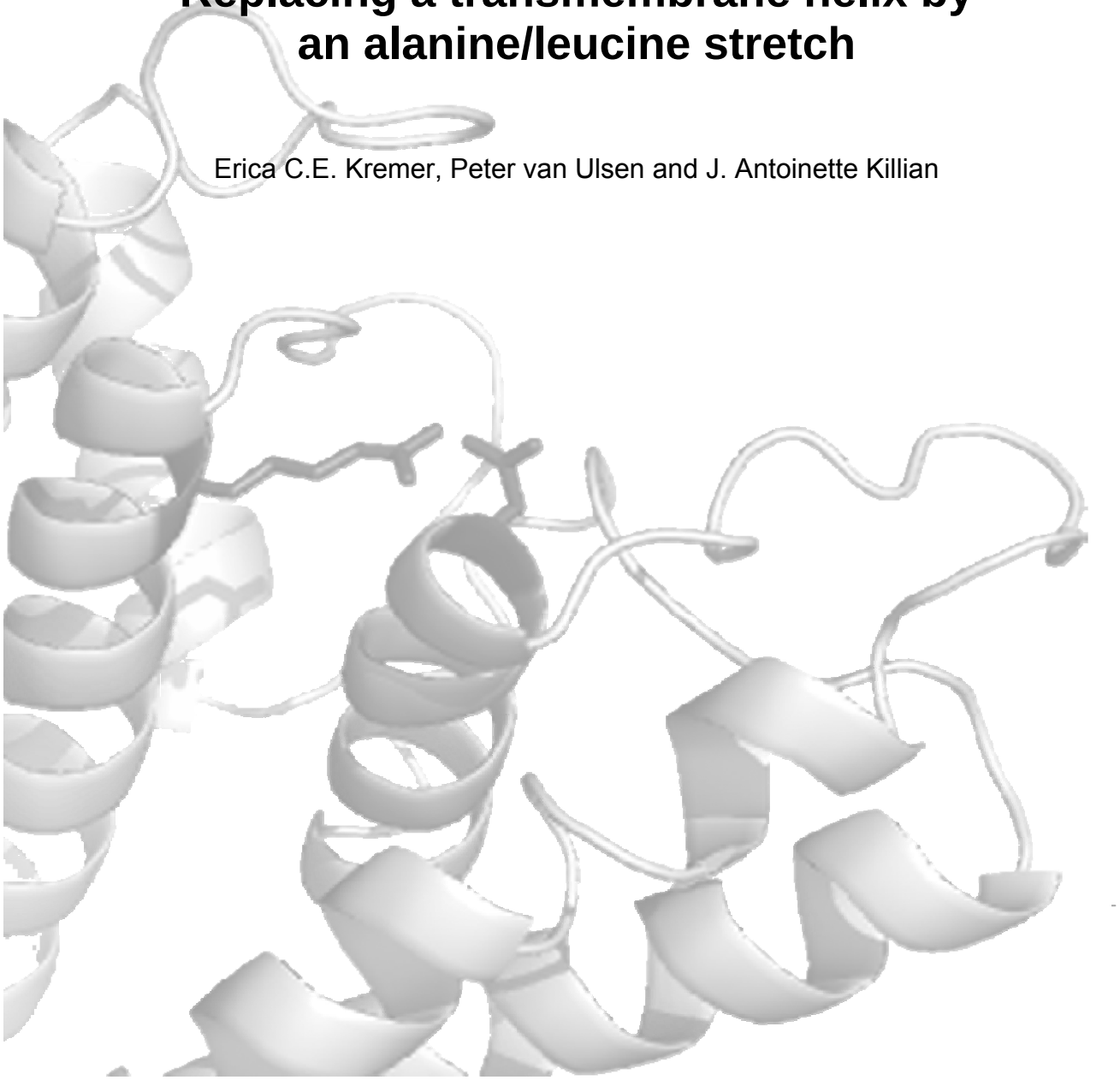
Appendix chapter 2

Replacing a transmembrane helix by an alanine/leucine stretch

Erica C.E. Kremer, Peter van Ulsen and J. Antoinette Killian



Abstract

In this study we show an adapted PCR method which allows to change up to 25 amino acids in two steps. This method is convenient when a significant part of a protein needs to be replaced by a non natural amino acid sequence. In our study this approach is used to replace the outer helix of the potassium channel KcsA by an alanine/leucine stretch.

Introduction

In microbiology it is a common approach to alter amino acids of a protein to investigate function or other properties of this protein. Two widely-used techniques to perform mutagenesis are the quick change mutagenesis kit and the restriction/ligation approach. The quick change kit is very convenient if one to three amino acids close together need to be altered. The restriction ligation approach is suitable if a large part of the protein needs to be replaced. Nevertheless this is only possible if the protein part that needs to be inserted is available as a DNA strand, since this method is performed on DNA level. However, we wanted to replace a transmembrane helix (TMI) of the potassium channel KcsA by a non-natural amino acid sequence; an alanine/leucine stretch. The reason for this replacement is the desire to study the influence of transmembrane helix content in KcsA tetramer stability. The inspiration to chose the alanine/leucine stretch as a replacement comes from studies on synthetic peptides containing an alanine/leucine stretch of which the properties and lipid interactions have been extensively studied [1-4]. This alanine/leucine stretch allows systematic studies on protein-lipid interactions e.g. by changing length or hydrophobicity of this stretch. The desire to make this KcsA mutant led us to set up an alternative PCR protocol which enables us to make this replacement in a convenient way.

Materials and methods

Materials

The following chemical reagents were purchased at Fermentas: Taq polymerase, dNTP's, MgCl and restriction enzymes. Primers with sequences indicated below were obtained from Biolegio:

Primer A: 5'CACTCCGCTATCGCTACGTGACTGGGT3'

Primer D: 5'CAGGATCCGATAAGCTTGGGCTGCAGGTCGACGGGT3'

Primer C: 5'GCGCTCGCTCTCGCGCTCGCACTAGCACTAGCGCTGGCTGAG CGCGGCGCACCGGGCGCGCA3'

Primer B: 5'GCGAGCGCGAGAGCGAGCGCCAGTGCGAGTGCTAGCGCTAG A GCCAGTGCCCTCCAGTGCAGCGCACTGCCGT3'

Primer design

Primers were designed according to generally accepted rules, e.g. 50-60% of C+G content, 3' end with C or G and a melting temperature (Tm)

between 60-80 °C. The idea is that primer A can anneal to the KcsA-WT sequence upstream from the mutation site and that it contains a unique restriction site, XbaI in this case. Primer D does the same, only on the complementary strand of the DNA and with BamHI as restriction site. These restriction sites are necessary to ligate the desired fragment back into the expression vector at the end of the protocol. Primer B is designed so that it partly anneals with the KcsA-WT DNA sequence and partly consist of the sequence that needs to be introduced, alanine/leucine in our case. Primer C is designed to do the same only on the complementary DNA strand *(Fig. 1 step 1).* In the middle, primer B and C should have an overlapping sequence of at least ten amino acids so that both introduced sequences can be combined in a later stage *(Fig. 1 step 3).* The latter feature makes that these primers have a length of around 65 bases which is exceptionally long.

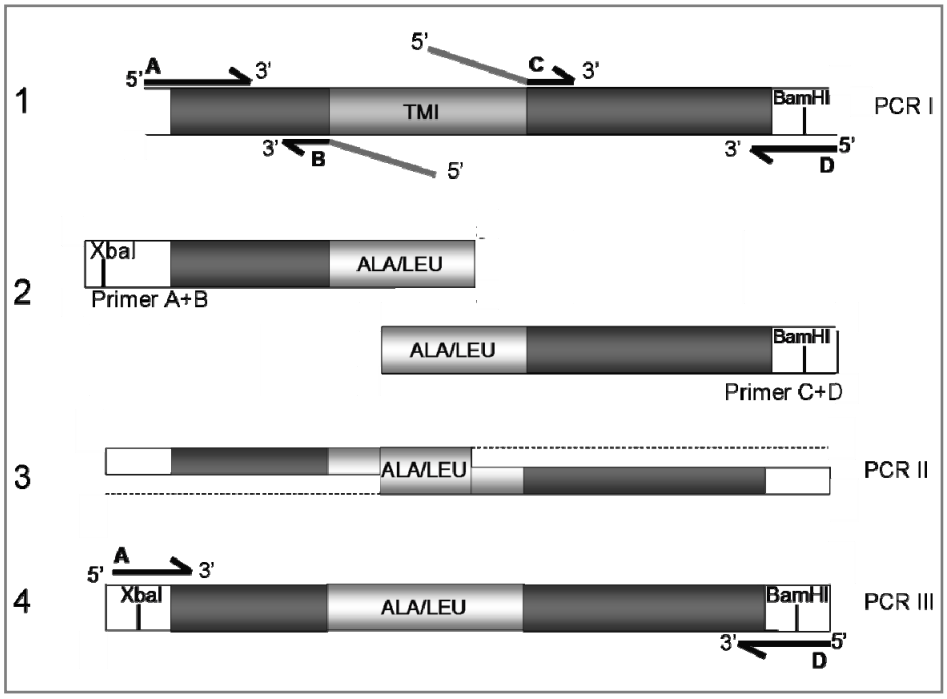


Figure 1: Schematic representation of PCRs performed to create KcsA-TMI and KcsA-TMII from KcsA-WT. Primers were designed as described in materials and methods (step 1). In PCR I two fragments are made with pT7-837KcsA-WT as template. One is made by primer A+B (200 kDa) and one by C+D (600 kDa) (step 2). These fragments are used as templates for PCR II in which some of the two fragments to dissociate and anneal to each other (step 3). At the end of PCR II primers A and D are added to perform PCR III which allows the elongation of the annealed fragments thereby introducing the alanine/leucine stretch instead of the original helix sequence (step 4). The unique BamHI and XbaI restriction sites are used to digest the desired fragment out of pCRII-TOPO and subsequently ligate it into the pT7-837 expression vector.

PCR

In PCR I two fragments are made with pT7-837KcsA-WT as template *(Fig. 1 step 2).* One is made by primer A+B and one by C+D. Thirty-five PCR cycles were performed with an annealing temp of 65°C. The two created fragments both contain the KcsA-WT DNA sequence (A+B N-terminal part and C+D C-terminal part) extended with mayor part of the alanine/leucine stretch. These fragments were checked for their correct length on a DNA gel and excised with the Promega gel extraction kit. After extraction, part of the product was ligated into the pCRII-TOPO vector for sequencing.

After sequence verification the remainder of the extracted fragment DNA was used as templates for the next PCR round. In PCR II, ten rounds with a annealing temp of 65°C were performed without the presence of KcsA-WT template, primer or polymerase (total volume 50µl). This allows some of the two fragments to dissociate and anneal to each other *(Fig. 1 step 3).* At the end of this PCR, primer A and D together with PCR reaction mix was added (25 µl) to the reaction tube for another thirty-five PCR cycles with an annealing temperature of 65°C. PCR III allows the elongation of the annealed fragments thereby introducing the alanine/leucine stretch instead of the original helix sequence *(Fig. 1 step 4).* The PCR product was verified on a DNA gel, the band of correct size was excised and it was subsequently ligated into pCRII-TOPO for sequencing. After DNA verification the desired fragment was digested out of pCRII-TOPO and ligated into the pT7-837 expression vector by making use of the unique BamHI and XbaI restriction sites.

Results

After some optimization of PCR conditions for PCR I fragments of the desired size (200 and 600 kDa) were obtained *(Fig. 2).* These fragments were successfully run on a DNA gel, extracted from this gel and used as template for PCR II which again after some optimization resulted in a fragment of the expected 800 kDa *(Fig. 3).*The ten amino acids overlap between the fragments from PCR I is too small to cause a visible shift in the obtained product of PCR II on DNA gel, which therefore remains 800 kDa.

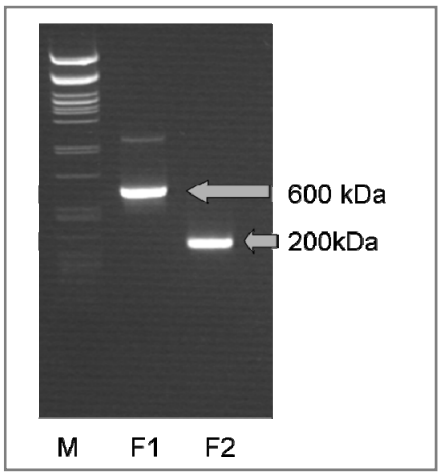


Figure 2: DNA-gel showing products of PCR I for fragment I (F1) created by primer A+B and fragment II (F2) created by primer C+D. Bands indicated with an arrow had the expected size and were excised from gel. M indicates the molecular weight marker.

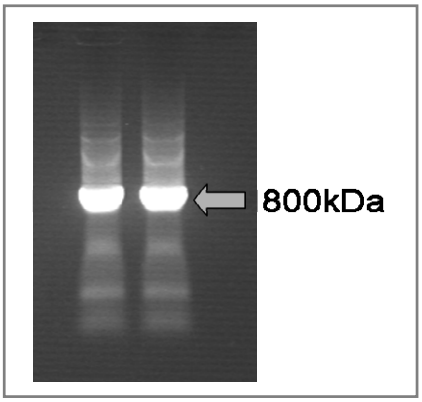


Figure 3: DNA-gel showing the product of PCR II. The band of expected size is indicated with an arrow and excised from gel for sequencing.

After sequence verification it was established that with this method TMI of KcsA was successfully replaced by an alanine/leucine stretch. Subsequently the altered (part of the) protein could be used to replace the KcsA-WT TMI in the pT7-837 expression vector, by making use of the BamHI and XbaI restriction sites which are present in both sequences. Moreover this vector was used to express the mutant protein in *E.coli.* With the same approach also TMII of KcsA could be replaced by such a stretch (results not shown).

Discussion

In summary we have shown that with our adapted PCR approach we have developed a complementary method to introduce a large number of mutations in a protein. This method is quicker than mutating more than six amino acids by quick change mutagenesis. However, conventional digestion/ligation methods would be much faster than our approach. The advantage of our approach over this latter approach is that there is no need for a DNA template of the desired insertion. This means that virtually any desired amino acid sequence, natural or artificial, can be introduced at any location in an existing protein sequence. In retrospect it has to be mentioned that a lot of precision should be attained in the primer design since small errors can lead to incorrect annealing, hairpin formation and point mutations. Furthermore optimizing the PCR conditions might take some endurance as for most (unusual) PCR reactions. In addition it needs to be mentioned that ordering the synthetic gene of desire at a specialized company makes a good, but probably more expensive alternative to our approach.

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Chapter 3

The molecular nature of KcsA tetramer stabilization by phosphatidic acid

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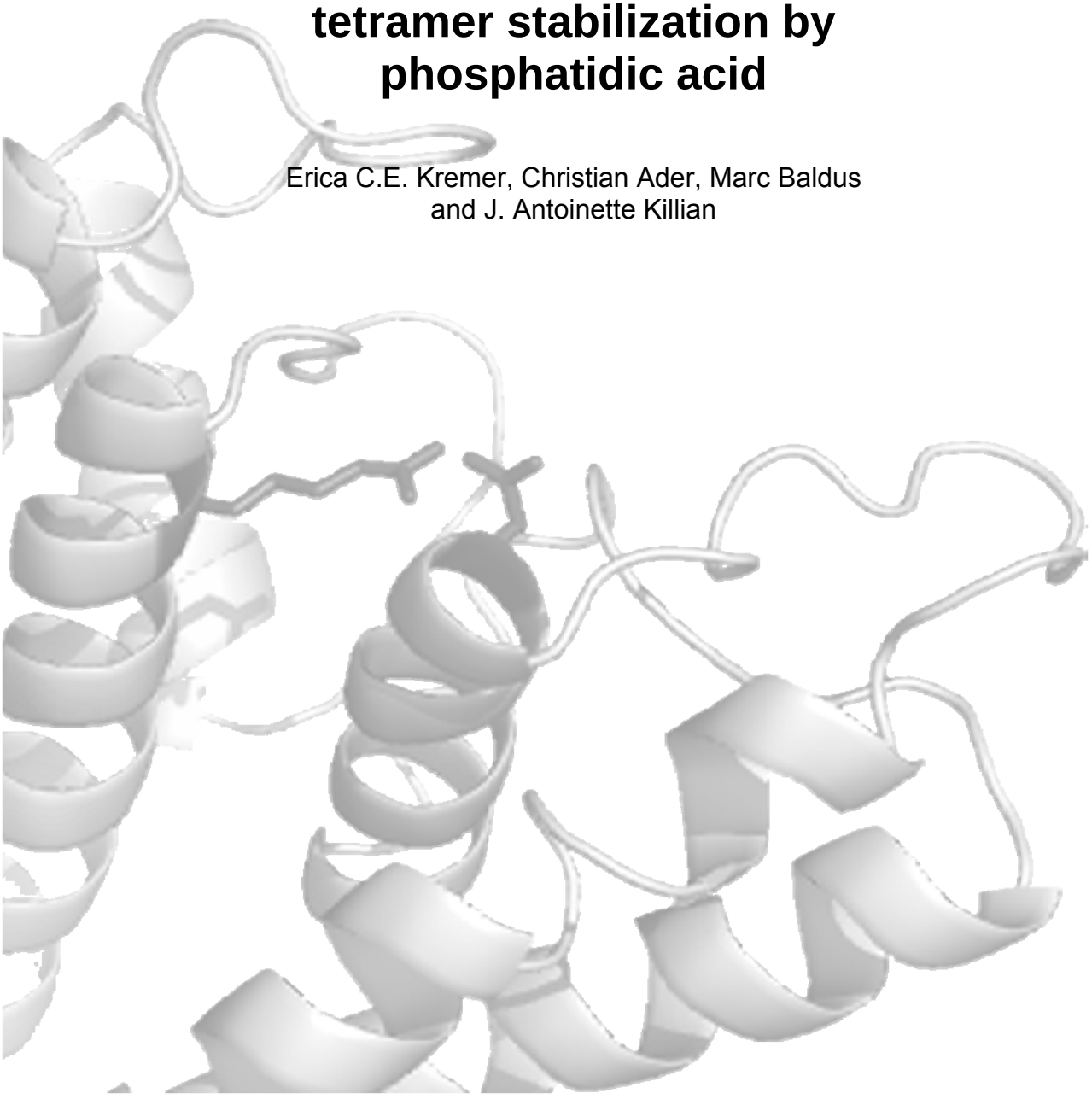

Abstract

The potassium channel KcsA from *Streptomyces lividans* forms a stable homotetramer which can be detected with SDS-PAGE. Previously it was found that the presence of the anionic lipid phosphatidic acid (PA) increases KcsA tetramer stability [1]. In this study we use a trifluoroethanol (TFE) titration to investigate the molecular nature of this stabilizing effect. We show that at low pH, were PA is more neutral, the stabilizing effect of PA on the KcsA tetramer is lost, indicating that the interaction most likely is charge dependent. Additionally we found that the chimeric channel KcsA-Kv1.3, in which the net charge of the loop between the outer and pore helix is significantly altered, is not stabilized by PA. This implies that the PA binding site lies within this altered region.

Introduction

For many membrane proteins it is known that they function in stable oligomeric structures [2, 3]. There are several evolutionary explanations for oligomer formation e.g. improvement of genetic efficiency, regulation of function and enhancement of stability [4]. For the potassium channel KcsA from the bacterium *Streptomyces lividans*, especially the latter two factors, function and stability, have been studied in relation to oligomerization [2, 5-8]. KcsA functions as a tetrameric assembly of four identical subunits which together form a potassium selective channel in the membrane. This homotetramer exhibits extreme stability, such that even when KcsA containing membranes are subjected to temperatures up till 90°C in SDS the protein still remains in the tetrameric conformation [9].

Interestingly, both stability and function have been found to be sensitive to membrane lipid composition [7, 9, 10]. It was observed that in the KcsA crystal structure tightly bound lipids, presumably PG, are present [11]. The binding of anionic lipids to the tetramer was found to be necessary for KcsA function [12] and it has been proposed that a hydrophobic pocket between two adjacent monomers forms the interaction site for these tightly bound lipids with the tetramer [11, 13]. Based on molecular dynamics simulations [13], fluorescence spectroscopy experiments [14] and mass spectrometry studies [15] it has been postulated that PG but in particular PA have a strong affinity to bind to this pocket. It is likely that binding of these lipids also affects the stability of the tetrameric conformation.

Previously it was found that PA and PG both can stabilize the tetramer, but that the presence of PA stabilizes the KcsA tetramer more compared to PG [1]. In this study we assess the molecular nature of this stabilizing interaction of PA with the KcsA tetramer. Purified KcsA-WT was reconstituted into different lipid bilayers and tetramer stability was tested at pH 7.4. We found that the stabilizing effect of PA on tetramer stability is specific.

Similar experiments performed at lower pH suggest that the interaction between KcsA and PA depends on the charge of PA. The importance of electrostatic interactions is further supported by experiments with the chimeric protein KcsA-Kv1.3, in which the loop between the outer helix and pore helix is altered.

Materials and methods

Materials

The following chemical reagents were purchased either from Fluka, Merck or Sigma Aldrich, available in the highest purity: Lysozyme, HEPES, KCl, NaCl, imidazole and isopropyl-β-D-thiogalactopyranoside (IPTG). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and *tetraoleoyl-cardiolipin (TOCL)* were purchased from Avanti Polar Lipids Inc. For clarity, the names of these lipids are abbreviated to PC, PG, PA, PE and CL respectively. n-Dodecyl-β-D-maltoside (DDM) was obtained from Anatrace Inc, Ni^{2+}-NTA agarose from Qiagen, trifluoroethanol (TFE) from Merck and Coomassie Brilliant Blue G-250 from ICN Biomedicals. The electrophoresis setup, proteins markers and Bio-Beads SM-2 adsorbent were purchased at Biorad Laboratories B.V. and the monoclonal anti-polyhistidine-peroxidase from Sigma Aldrich.

Protein expression and purification

KcsA expression and purification, with some minor adaptations, was performed as described previously in [9]. Briefly, KcsA was expressed with an N-terminal His-tag from pT7-KcsA in *Escherichia coli* strain BL21 (λDE3) upon IPTG induction. KcsA was purified in a buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM KCl. The membranes were solubilized in 3 mM DDM and incubated with pre-washed Ni^{2+}-NTA agarose beads overnight at 4°C. The bound His-tagged proteins were eluted with 300 mM imidazole pH 7.5 and 1 mM DDM. The purity of proteins was assessed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The presence of DDM precluded the use of the Bradford assay, and therefore the protein concentration was assessed by SDS gel after staining with Coomassie Blue, using a standard of bovine serum albumin (BSA).

Vesicle preparation and protein reconstitution

This assay was performed as described in [1]. Briefly, large unilamellar vesicles (LUVs) were prepared by extrusion through 200 nm filters. LUV's (5 mM phospholipids) were prepared in vesicle buffer (10 mM HEPES, pH 7.5, 45 mM NaCl, 5 mM KCl or citrate buffer pH 4.0 or 5.0 containing 45 mM NaCl and 5 mM KCl, solubilized with 1% Triton X-100 (w/v) and mixed with DDM solubilized KcsA proteins at a 1:2000 protein:lipid molar ratio. The detergent was removed using pre-washed Bio-Beads. The reconstituted vesicles were collected by centrifugation (1 hour, TLA 100.1 rotor, 100000 rpm or 434902 x g, and 4°C). The proteoliposomes were finally resuspended in one of the buffers described above. KcsA-Kv1.3 reconstituted in different lipid vesicles was a kind gift from the Pongs lab [16].

SDS-PAGE stability assay

This assay was performed as previously described in [1]. Briefly, membrane fractions of cells overexpressing KcsA, or purified protein in DDM or vesicles were divided in equal aliquots of 10 µl containing approximately 2,5 µg

of protein and incubated with increasing amounts of TFE for one hour at room temperature. TFE was added in an aqueous solution with a total concentration of 5 mM KCl, 45 mM NaCl to minimize changes in ionic strength. The TFE containing samples, with a total volume of 13 µl, were mixed with 5 µl of an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8, 50% glycerol, 0.01% bromophenol blue and 10% SDS) and directly run on 15% acrylamide gel in the presence of 0.1% SDS at room temperature. Afterwards proteins were visualized either by Western blot in combination with an α-His antibody for the *in vivo* samples or with Coomassie Brilliant Blue staining for the purified proteins. The gels containing purified protein samples were scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The tetramer band intensity present at 0 vol% TFE was set to 100%. The amount of tetramer (%) was plotted against the TFE concentration (vol%) for the stability assay.

Results

Lipid specificity of KcsA tetramer stabilization

Previous studies indicate that PA has a specific stabilizing interaction with the KcsA tetramer [1]. To investigate the molecular details of this interaction, the stability of purified KcsA-WT was tested in different lipid bilayers upon TFE incubation. Since the main features of PA are its small headgroup in combination with its charge, the used bilayers were chosen in such a way that the role of these features in stability could be assessed. The lipids used thus differ in shape and charge of the headgroups, whereas the tail region is unaltered. Lipid vesicles containing KcsA-WT were incubated with increasing vol% of TFE for one hour at pH 7.4 and the intactness of the tetramer was visualized on a 15% SDS gel with Coomassie Brilliant Blue staining (*Fig. 1A*).

From the gels we can conclude that the KcsA-WT tetramer is stable up till 20-30 vol% TFE in all lipids whereas in PC/PA a light tetramer band is still visible at 50 vol% TFE. This stabilizing effect of PA is even more evident when the tetramer bands of several experiments are quantified and plotted against TFE concentration (*Fig. 1B).*

It has been proposed that the interaction of PA with the KcsA tetramer is dependent on the positive charges present on the N-terminus [1]. To investigate whether indeed the N-terminus plays a role we performed similar experiments on a mutant lacking the 16 N-terminal amino acids (KcsA-ΔN). In the presence of PA KcsA-ΔN *(Fig. 2)* appears to be even slightly more stable compared to KcsA-WT *(Fig. 1B),* suggesting that the N-terminus is not involved in stabilization by PA. On the other hand the general tetramer stability was found to be lower for KcsA-ΔN (10-25 vol% TFE) (*Fig. 2*) compared to that of KcsA-WT (20-30 vol% TFE) *(Fig. 1)* in the other lipid systems. This makes sense since KcsA-ΔN is lacking the amphipathic helix that binds to the membrane interface and removal of this helix will thus decrease the interaction of KcsA with the membrane [15, 17]. Taken together this indicates that the N-terminus of the protein plays a role in the general stability of the KcsA tetramer through its membrane interaction, but that it is not involved in the PA associated increase in tetramer stabilization.

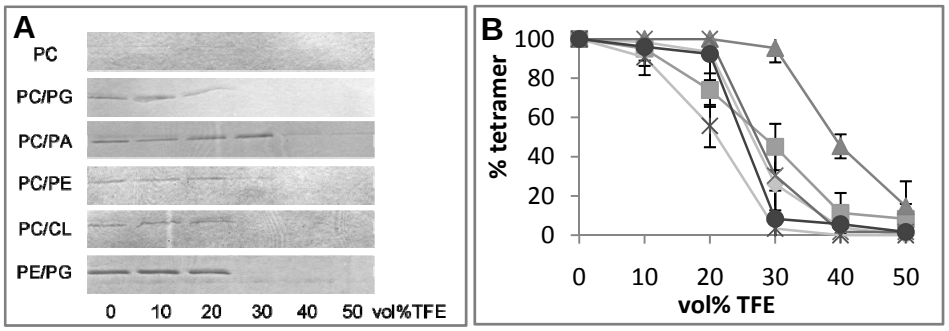

Figure 1 (A) Representative SDS-gels of KcsA-WT tetramer stability in different lipid bilayers. Each mixed bilayer had a 7:3 molar ratio except PC/CL which had a phosphate based ratio of 7:3. (B) Tetramer stability of KcsA-WT in different bilayers: PC (♦), PC/PG (■), PC/PA(▲), PC/PE (x), PC/CL (→) and PE/PG (●). All experiments were performed in a buffer containing 5 mM KCl and at pH 7.4. For each lipid/protein combination at least three fully independent experiments were performed.

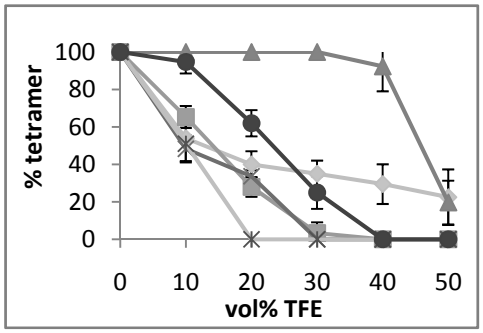

Figure 2: Tetramer stability of KcsA-ΔN in different bilayers. PC (♦), PC/PG (■), PC/PA(▲), PC/PE (x), PC/CL (→) and PE/PG (●). All experiments were performed in a buffer containing 5 mM KCl and at pH 7.4. For each lipid/protein combination at least three completely separate experiments were performed.

The role of the charge state of PA in KcsA stabilization

From the above we can conclude that the effect of tetramer stabilization by PA is indeed specific for this lipid and not associated with the presence of the N-terminus. To see if this interaction depends on the charge of PA, similar experiments as described above were performed, but now at lower pH. By lowering the pH to 4.0 the charge state of PA will change from negative to nearly neutral (pKa = 4.4). *Fig. 3A* shows that at pH 4.0 the stabilizing effect of PA totally disappears suggesting that the charge state of PA is an important factor for tetramer stabilization. However at pH 5.0 still some of the increased stability is present. The shape of the stability curve at pH 5.0 is remarkable and seems to consist of two populations. At 10 vol % TFE 25% of tetramer is already lost, indicating that this population is less stable as compared to pH 7.4. However the remaining 75% has similar stability compared to pH7.4. This will be discussed later.

For KcsA-WT in PC/PG no change in tetramer stability was observed upon lowering the pH *(Fig. 3B)* and the same result was obtained in all of the other lipid compositions tested *(data not shown).* Moreover, also for KcsA-ΔN similar behavior was found at pH 7.0 and pH 4.0 *(data not shown),* indicating that the change of pH itself does not alter the general tetramer stability and hence that the charge state of PA most likely is a defining factor in tetramer stabilization. However it can not be ruled out that additional factors, such as conformation, are also altered by the decrease in pH and thereby influence tetramer stability in the presence of PA.

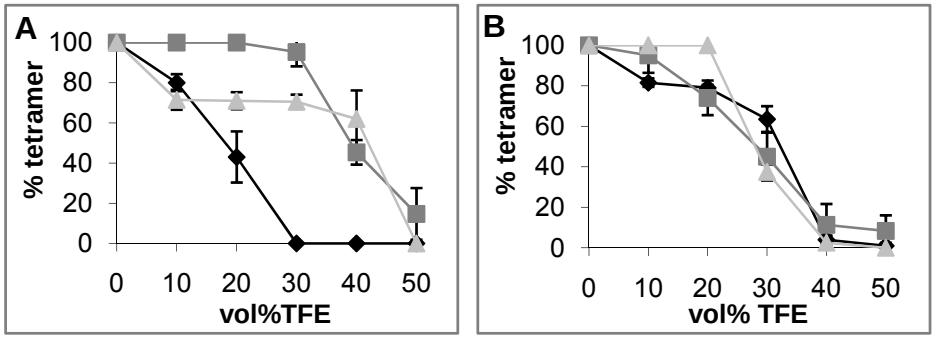

Figure 3: KcsA-WT in PC/PA (A) and PC/PG (B) at different pH. pH 4.0 (♦), pH 5.0 (▲) and pH 7.4 (■). All experiments, at least three per data point, were performed in a buffer containing 5 mM KCl.

Tetramer stability in KcsA-Kv1.3

So far we have established that the stabilizing effect of PA on the KcsA-WT tetramer is lipid specific and most likely charge dependent, but that it does not depend on the presence of the N-terminus of the protein. The next step is to identify the binding site of PA to the KcsA-WT tetramer. It has been postulated that the lipid found in the crystal structure represents PG, because this lipid was copurified with KcsA [11]. However, in principle it could also represent other lipids, such as PA, which because of their low abundance are less likely to be detected by Thin Layer Chromatography. It has been found that residue R64 of one monomer and R89 of an adjacent monomer together are the main amino acids involved in binding of specific lipids [10, 11, 13, 14, 18]. Furthermore it has been suggested that at least three of these specific lipid binding sites need to be occupied for channel functioning [12]. Simulation studies indicate that PG as well as PE and PA are able to bind to this pocket with a reasonable affinity [13]. To investigate whether this proposed pocket for tightly bound lipids is indeed the interaction site by which PA stabilizes the KcsA-WT tetramer, we made use of the chimeric potassium channel KcsA-Kv1.3 [19]. This chimera, of which the structural properties already have been extensively studied on a molecular level [20, 21], has eleven amino acids in the loop between the outer- and pore-helix which are different from KcsA-WT, as shown in *Fig. 4A and B.* Within KcsA this loop is thought to be involved in stabilizing the selectivity filter and part of the protein that forms the interface between monomers and therewith the proposed lipid binding pocket. Especially mutations causing

changes in charge *(Fig. 4A and B),* are expected to interfere with the interaction between PA and the tetramer, thereby inhibiting tetramer stabilization by PA. Indeed, *Fig. 5* shows that KcsA-Kv1.3 has similar tetramer stability in PC, PC/PG and PC/PA. Moreover, the general tetramer stability of KcsA-Kv1.3, with a dissociation between 10-30 vol% TFE (*Fig. 5*), is lower compared to KcsA-WT, with a dissociation around 20-30 vol% TFE (*Fig. 2*).

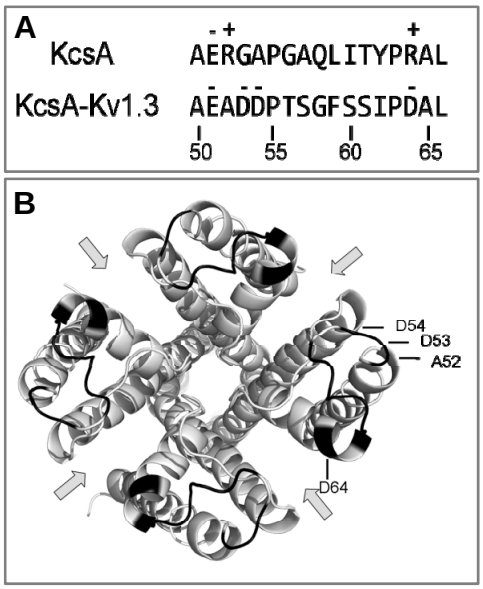

Figure 4: Differences in amino acid composition between KcsA-WT and KcsA-Kv1.3. (A) The amino acids of both proteins with the charge indicated above the sequence whereas the amino acid number, as in KcsA-WT, is given below the sequence. (B) Cartoon, based on PDB 3EFF (Uysal et al 2009), of the top view of the KcsA-Kv1.3 tetramer (white) with mutations compared to KcsA-WT indicated in black. Residues that are altered in their charge are assigned in one monomer and the lipid binding pockets between adjacent monomers are indicated with arrows.

We propose that a slightly different oligomeric conformation of KcsA-Kv1.3 is responsible for its lower stability as compared to KcsA-WT in the presence of all lipids, and in particularly of PA. This seems to be supported by the presence of very prominent KcsA-Kv1.3 dimers in the TFE dissociation assay on SDS-gel *(Fig. 6).* These KcsA-Kv1.3 dimers are present in all bilayers (data not shown) and do not seem sensitive to TFE concentrations up till 50 vol%. These remarkably stable dimers are most likely essential for the formation of a functional tetramer and probably are induced by an allosteric effect in which binding between two adjacent monomers, possibly mediated by lipids, hinders binding of the monomers of this dimer to the monomers of the other dimer.

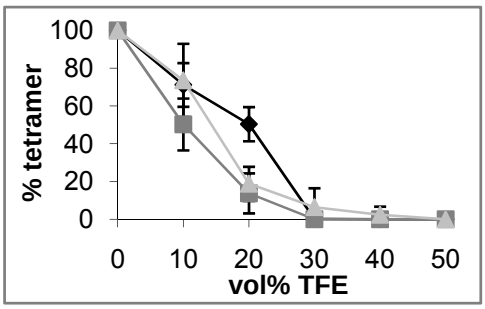

Figure 5: Tetramer stability of KcsA-Kv1.3 in different lipid bilayers. PC (♦), PC/PG (■) and PC/PA (▲). All experiments, at least three per data point, were performed in a buffer containing 5 mM KCl and at pH 7.4.

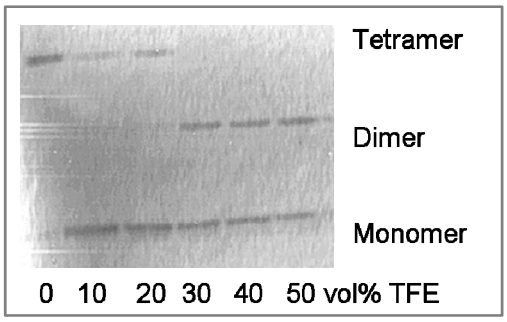

Figure 6: Representative SDS- gel of TFE tetramer dissociation of KcsA-Kv1.3 showing tetramers, dimers and monomers. In this case the lipid bilayer consisted of pure PG. This experiment was performed in a buffer containing 5 mM KCl and at pH 7.4.

Discussion

In this study we show that PA has a strong stabilizing effect on KcsA-WT tetramer stability. The interaction that lies at the basis of this increase of stability is lipid specific and most likely depends on the charge of PA since at low pH no stabilization effect by PA was found. Given that the chimeric channel KcsA-KV1.3, which has mutations in and around the proposed lipid biding site, does not show any stabilization by PA, we propose that the interaction site of PA lies within this pocket. Below the possible factors involved in tetramer stabilization by PA will be discussed in more detail.

Lipid specificity in KcsA tetramer stability

The stabilizing effect of PA on the KcsA-WT tetramer was found to be specific for this lipid. Simply the combination of the presence of a negative charge and a small lipid head group is not sufficient to explain this effect of PA, since neither PC/CL nor PE/PG bilayers which also contain both these features, albeit to a much lesser extent, show a significant stabilizing effect. However, PA is an exceptional lipid since it basically lacks a lipid headgroup, thereby minimizing steric hindrance, and additionally its phosphate group has a higher charge density than all other phospholipids. The unique combination of charge and hydrogen bonding properties of the phosphate group of PA, which both enhance its negative charge, results in increasing strength of interactions with positively charged protein side chains [22]. This special feature could be of significant importance for the binding of PA to the KcsA tetramer.

Although it certainly is remarkable that PA has such a huge effect on tetramer stability, it has to be noted that in our experiments around 30 mol% PA is present. This is significantly more than the small amount of PA that is present in biological membranes [23]. In previous studies it was found that only at concentrations above 20 mol% of PA the KcsA-WT tetramer stability is increased in PC/PA compared to PC/PG. This indicates that the binding of PA to the KcsA-WT tetramer and its stabilizing effect are less likely to occur *in vivo*, suggesting that perhaps strong stabilization of the KcsA tetramer is unfavorable for channel functioning.

Role of low pH in KcsA tetramer stability

It was found that the interaction between PA and the KcsA tetramer is dependent on the charge state of PA. This was determined by lowering the pH from 7.4 to 4.0 in the TFE stability assays, thereby changing the charge of PA from negative to neutral [22]. At pH 4.0 no stabilization by PA was observed

indicating that the loss of charge causes a loss of specific interaction. However at pH 5.0 an intermediate stability was found, matching with the decrease in negative charge of PA. This decrease causes around 25% of the tetramers to be less stable compared to pH 7.4 whereas the remaining 75% seems to be just as stable compared to pH 7.4. This could fit with the change in pKa that will cause around 25% of the PA molecules to have already lost their charge at pH 5.0.

However, an alternative possibility for the decrease in tetramer stability at low pH could be the opening of the channel, since it has been reported that KcsA gates at acidic pH [24-26]. Opening of the channels could give rise to the loss of the specific interaction of PA with the tetramer and thereby decreasing the tetramer stability. Suggesting that PA and maybe also other lipids, can only stabilize the channel in its closed conformation. This would agree with the observation that lipid binding to at least three of the lipid binding sites of a closed channel is necessary to enable the channel to open [12]. When the open conformation is obtained the lipids then may lose their (stabilizing) interaction with the tetramer thereby enabling the channel to regain its closed conformation. We conclude that the unique combination of charge and small headgroup, enhanced by its hydrogen bonding capacity, is responsible for the special role of PA in tetramer stabilization. However the conformation of the protein may further modulate the interaction with PA.

The molecular nature of the specific interaction of KcsA and PA

By studying the chimeric protein KcsA-Kv1.3 in a similar lipid dependent way as done for the KcsA-WT it was observed that PA is not able to increase tetramer stability in this protein. This indicates that the PA binding site in KcsA-WT is most likely harbored by the region which is mutated in KcsA-Kv1.3. It has been postulated that R64 together with R89 forms the basis of a pocket able to bind specific anionic lipids [13]. The mutation of residue R64 into a negatively charged aspartic acid in the chimeric protein KcsA-Kv1.3 therefore is an appealing cause for the lost interaction with PA *(Fig. 4).* To determine whether this amino acid indeed lies at the basis of the specific interaction between PA and the KcsA-WT tetramer it would be interesting to prepare the R64D mutation in KcsA-WT and test the effect on tetramer stability and on lipid specificity. Furthermore such a specific mutation could provide more insight concerning the possibility that this suggested binding site is involved in tetramer stability as well as gating.

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Chapter 4

The role of amino acid residue R64 in KcsA tetramer stability and lipid binding

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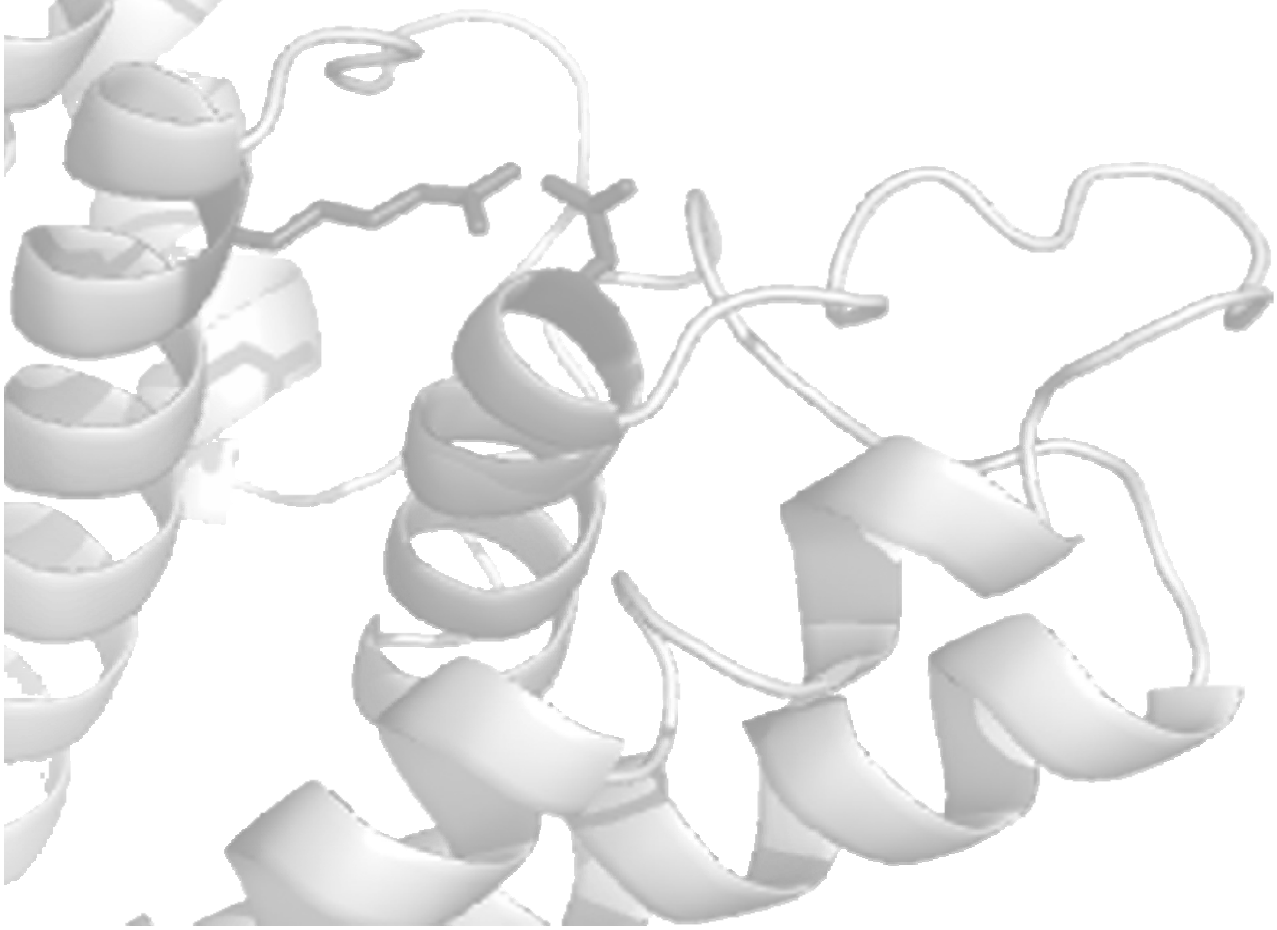

Abstract

In this study we investigated the role of the basic amino acid residue R64 in stability and lipid binding of the tetrameric potassium channel KcsA. R64 is part of the proposed lipid binding site and was suggested to be involved in the strong tetramer stabilizing effect of the anionic lipid phosphatidic acid (PA) via electrostatic interactions *(previous chapter)*. Substitution of this basic residue into an acidic acid can be expected to interfere with binding of anionic lipids, leading to a decreased stability. However here we observed the opposite. We found that substituting residue R64 by an aspartic acid leads to a large increase in tetramer stability in a variety of mixtures of synthetic lipid bilayers, including a PA containing mixture. Substitution by alanine had only a small stabilizing effect. The stability enhancement in KcsA-R64D is probably caused by a salt bridge that is formed between D64 and R89 from the adjacent monomer, substituting the bound lipid. The importance of lipid binding for tetramer stabilization in native KcsA is supported by mass spectrometry experiments in which the tetramer is found to specifically dissociate into dimers rather than monomers. Based on these measurements we propose that occupation of specific lipid binding sites within the KcsA tetramer influences the adjacent binding site via an allosteric effect.

Introduction

Many properties of the potassium channel KcsA from *Streptomyces lividans* are influenced by lipids, including assembly, stability and function [1-5]. These protein-lipid interactions take place at general and specific interaction sites. The general sites typically represent electrostatic interactions between charged KcsA residues facing the lipid bilayer and the charges present within the bulk of lipids. The influence of lipids on assembly and stability are likely at least in part caused by these electrostatic interactions, but also factors like hydrophobic thickness [6] and lateral pressure [2] of the membrane might play a role. In addition, based on crystallographic data [7, 8] and molecular modeling [9], specific binding sites are present, which are formed by a cleft that is located on the interface of two adjacent monomers governing specific protein-lipid interactions. The tightly bound lipids occupying this cleft in the KcsA crystal structure [10] were identified as PG, based on the observation that PG could be copurified with KcsA. However, this does not exclude the possibility that other, less abundant lipids bind to the same position. The main residues involved in lipid binding were identified as R64 from one monomer and R89 from the adjacent monomer and it was found that the binding affinity has the following preference PA > PG >> PE [9, 11, 12]. The proposed high affinity binding of PA is experimentally supported by the observation that PA has a specific stabilizing effect on the KcsA-WT tetramer whereas PG only has a weak stabilizing effect [13]. Additionally it was shown that anionic lipids are necessary for function which may be related to the preference of binding and/or their effect on stability [10, 14].

Another interesting feature of the lipid binding sites is that, based on tryptophan fluorescence quenching studies, at least three of the four sites need to be occupied by a lipid in order for the channel to open [15]. It is likely that the

binding of lipids to these pockets needs to be transient in order to allow gating, since it is believed to be necessary for at least one bound lipid to leave a binding pocket in order to facilitate closure of the channel [15].

In the previous chapter we found that PA is able to stabilize the KcsA-WT tetramer whereas for the KcsA-Kv1.3 tetramer no such stabilization was observed, indicating that (part of) the PA binding site must lie within the eleven amino acids altered in KcsA-Kv1.3 compared to KcsA-WT. These amino acids include residue R64 which in KcsA-Kv1.3 is replaced by an aspartic acid, thereby changing its charge from positive to negative. Since R64 is thought to be a key player in specific lipid binding of KcsA, it is possible that this mutation on its own is responsible for the lack of tetramer stabilization by PA in KcsA-Kv1.3. To further investigate the role of R64 in tetramer stability and lipid binding we mutated residue R64 in KcsA-WT into an alanine (R64A) or into an aspartic acid (R64D) as in KcsA-Kv1.3 and tested its stability in a variety of lipid mixtures.

We will show that both KcsA-R64 mutants have a significantly altered tetramer stability compared to KcsA-WT. A model is proposed in which the amino acid at position 64 plays a crucial role in stabilizing the tetramer, either by direct electrostatic interactions between subunits or by lipid mediated monomer-monomer contacts.

Materials and Methods

Materials

The following chemical reagents were purchased either from Fluka, Merck or Sigma Aldrich, available in the highest purity: Lysozyme, HEPES, KCl, NaCl, imidazole and isopropyl-β-D-thiogalactopyranoside (IPTG). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and *tetraoleoyl-cardiolipin (TOCL)* were purchased from Avanti Polar Lipids Inc. For clarity, the names of these lipids are abbreviated to PC, PG, PA, PE and CL respectively. n-Dodecyl-β-D-maltoside (DDM) was obtained from Anatrace Inc, Ni^{2+}-NTA agarose from Qiagen, trifluoroethanol (TFE) from Merck and Coomassie Brilliant Blue G-250 from ICN Biomedicals. The electrophoresis setup, proteins markers and Bio-Beads SM-2 adsorbent were purchased from Biorad Laboratories, the monoclonal anti-polyhistidine-peroxidase from Sigma.

Protein expression and purification

KcsA expression and purification, with some minor adaptations, was performed as described previously in [4]. Briefly, KcsA was expressed with an N-terminal His-tag from pT7-KcsA in *Escherichia Coli* strain BL21 (λDE3) upon IPTG induction. After collection cells were lyzed in a French press to obtain membrane fractions for *in vivo* studies. Alternatively KcsA was purified in a buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM KCl. The solubilized membranes in 3 mM DDM were incubated with pre-washed Ni^{2+}-NTA agarose beads overnight at 4 °C. The bound His-tagged proteins were eluted with 300 mM imidazole at pH 7.5 and 1 mM DDM. The purity of proteins was assessed by sodium dodecylsulphate–polyacrylamide gel electrophoresis

(SDS–PAGE). The protein concentration was assessed by SDS gel after staining with Coomassie Blue, using a standard of bovine serum albumin (BSA).

KcsA mutagenesis

Point mutations in *kcsA* were introduced using the Quickchange mutagenesis kit according to the recommendations by the manufacturer. (Primer design and PCR were optimized by following the supplied protocol.) Pt7-837KcsA-WT was used as template DNA. Obtained mutations were verified by DNA sequencing.

Vesicle preparation and protein reconstitution

Large unilamellar vesicles (LUVs) were prepared by extrusion through 200 nm filters [13]. For protein reconstitution, LUV's (5 mM phospholipids) were prepared in vesicle buffer (10 mM HEPES, pH 7.5, 45 mM NaCl, 5 mM KCl or citrate buffer pH 4.0 or 5.0 containing 45 mM NaCl and 5 mM KCl, solubilized with 1% Triton X-100 (w/v) and mixed with DDM solubilized KcsA proteins at a 1:2000 protein:lipid molar ratio as described [13]. The detergent was removed using pre-washed Bio-Beads and the reconstituted vesicles were collected by centrifugation (1 hour, TLA 100.1 rotor, 100000 rpm or 434902 x g, and 4°C). The proteoliposomes were finally resuspended in one of the buffers described above.

SDS-PAGE stability assay

The tetramer stability assay was performed as previously described in [13]. Briefly, membrane fractions of cells overexpressing KcsA, or purified protein in DDM or vesicles were divided in equal aliquots of 10 µl containing approximately 2,5 µg of protein and incubated with increasing amounts of TFE for one hour at room temperature. TFE was added in an aqueous solution with a total concentration of 5 mM KCl, 45 mM NaCl to minimize changes in ionic strength. The TFE containing samples, with a total volume of 13 µl, were mixed with 5 µl of an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8,50% glycerol, 0.01% bromophenol blue and 10% SDS) and directly run on 15% acrylamide gel in the presence of 0.1% SDS at room temperature. Afterwards proteins were visualized either by Western blot in combination with an α-His antibody for the *in vivo* samples or with Coomassie Brilliant Blue staining for the purified proteins. The gels containing purified protein samples were scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The tetramer band intensity present at 0 vol% TFE was set to 100%. The amount of tetramer (%) was plotted against the TFE concentration (vol%) for the stability assay.

Tryptophan fluorescence

All fluorescence experiments were performed as described in [13]. Briefly, samples in a purification buffer at room temperature using a Varian Cary Eclipse spectrofluorometer and a 200 µl quartz cuvette. The samples were excited at 280 nm and emission spectra were collected between 300 and 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm. Spectra were corrected for imidazole contribution afterwards.

Mass spectrometry

KcsA was purified as described above. The purification buffer was exchanged 6 times in Biomax (10K 0,5 ml) centrifugal filters, with a 250 mM ammonium acetate buffer containing approximately 200 µM DDM, The tandem MS spectrum of wild-type KcsA was recorded with a high mass modified QToF mass spectrometer (Waters Manchester UK/Modefication MSVision Almere NL). Samples were introduced with gold coated capillairies into the nano-ESI source. The capillairy voltage was set to 1500 V and the sample cone was at 175 V. The 17+ ions at m/z 4160 of the tetramer were selected and submitted to the hexapole collision cell filled with Xenon up to 2.10^{-2} mbar at a collision energy of 100 V. The MS spectrum was recorded with an LCTmass spectrometer (Waters Manchester UK). Using the same conditions for the capillairy and sample cone as had been used for the QToF. This method was adapted from [16].

Results

Influence of R64 mutations on KcsA tetramer stability

To investigate the role of residue R64 in KcsA-WT tetramer stability, mutants were made in which residue R64 was replaced by an alanine or aspartic acid. Subsequently their *in vivo* tetramer stability was tested by incubating *E.coli* membranes containing KcsA, with varying amounts of TFE, followed by SDS-PAGE for visualization of the amount of KcsA tetramers. Surprisingly, we found that both R64 mutants are more stable *in vivo* compared to KcsA-WT, but that in particular the KcsA-R64D tetramer is highly stable, even till 50 vol% of TFE *(Fig 1).*

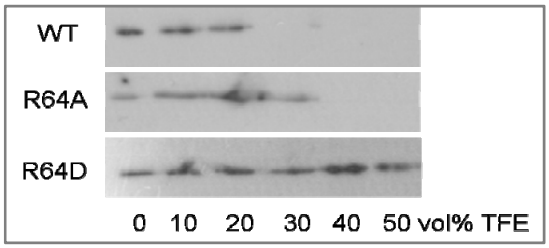

Figure 1: Representative SDS-gels for KcsA-WT, KcsA-R64A and KcsA-R64D tetramer stability upon TFE incubation in vivo. Proteins were detected by using an α-His antibody and the tetramer band runs at a molecular weight of 67 kDa.

Similar tetramer stabilities as obtained in *E.coli* membranes were found for the purified proteins in DDM, as shown in representative gels in *Figure 2A* and as quantified in *Figure 2B.* Again KcsA-R64D shows a significant increase in tetramer stability whereas KcsA-R64A only shows a slight enhancement in tetramer stability. These results indicate that both *in vivo* and *in vitro* residue R64 indeed is involved in tetramer stabilization, but that the R64D mutation increases stability rather than causing the destabilization that was expected based on the results obtained for KcsA-Kv1.3 (chapter 3).

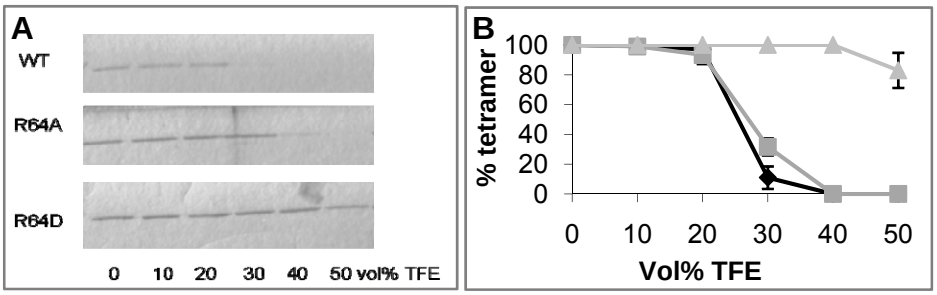

Figure 2: (A) Representative SDS-gels for KcsA-WT, KcsA-R64A and KcsA-R64D tetramer stability in DDM. (B) Graph showing tetramer stability in DDM upon a titration with TFE for KcsA-WT, KcsA-R64A and KcsA-R64D. The tetramer band runs at a molecular weight of 67 kDa. Each data point represents at least three experiments performed at 5 mM KCl and pH 7.4. KcsA-WT is indicated with the (♦), KcsA-R64A with (■) and KcsA-R64D with (▲).

Influence of R64 mutations on KcsA tryptophan fluorescence

As a complementary approach to the SDS-PAGE assay we also performed tryptophan fluorescence experiments on the purified proteins in DDM. Whereas KcsA-R64A showed a similar fluorescence spectrum as KcsA-WT, KcsA-R64D gave a small red shift at 0 vol% TFE *(Fig. 3A)*. This indicates that in KcsA-R64D the tryptophans are more exposed to a hydrophilic environment suggesting that replacing the positive charge of R64 by D64 causes rearrangements in the pore helix, by which W67, W68 and/or W87, which all are located in close proximity of R64, may become more exposed to the hydrophilic environment. Just deleting the positive charge in the KcsA-R64A mutant apparently does not result in such rearrangements, indicating that the negatively charged D64 might bring special conformational features to the protein.

Fig. 3B-D show emission spectra of the TFE titrations performed on KcsA-WT and KcsA-R64 mutants and *Fig. 3E* shows the emission maximum of the fluorescence as function of TFE concentration. The TFE titration performed on KcsA- WT *(Fig. 3B)* shows that between 10 and 40 vol% TFE a red shift of around 8 nm occurs, which has a midpoint around 23 vol% TFE *(Fig. 3E).* A similar shift is found for KcsA-R64A *(Fig. 3C),* but with a midpoint around 25 vol% TFE *(Fig. 3E).* KcsA-R64D shows a slightly larger red shift of around 10 nm *(Fig. 3D)* with a midpoint around 32 vol% of TFE *(Fig. 3E).*

For KcsA-WT and KcsA-R64A the red shifts found with tryptophan fluorescence roughly correlate with the percentage TFE found necessary for tetramer dissociation in the SDS-PAGE experiments. This suggests that the observed structural rearrangements within the protein lead to the dissociation into monomers. For KcsA-R64D the midpoint of the red shift occurs at a higher TFE concentration, but not as high as the concentration required for dissociation of the tetramer. This indicates that even though probably similar structural rearrangements occur as in KcsA-WT and KcsA-R64A, these are not sufficient to destabilize the KcsA-R64D tetramer in such a way that it dissociates into monomers.

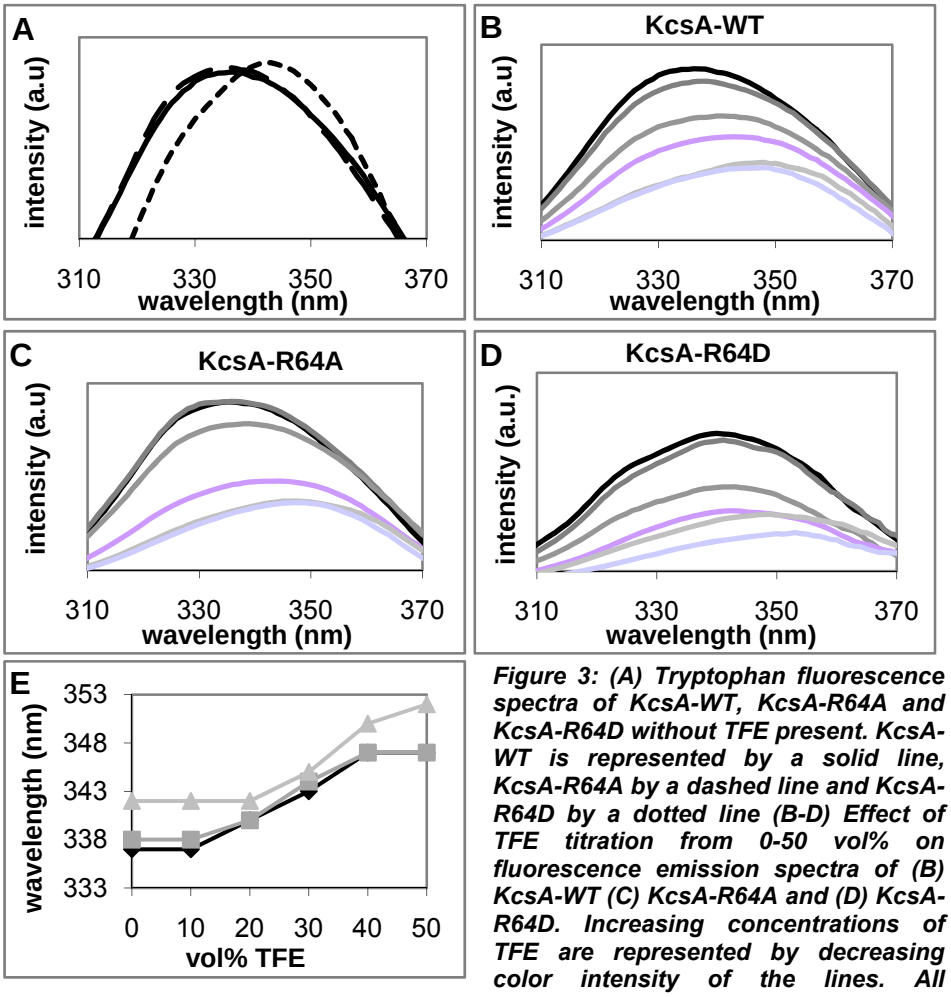

Figure 3: (A) Tryptophan fluorescence spectra of KcsA-WT, KcsA-R64A and KcsA-R64D without TFE present. KcsA-WT is represented by a solid line, KcsA-R64A by a dashed line and KcsA-R64D by a dotted line (B-D) Effect of TFE titration from 0-50 vol% on fluorescence emission spectra of (B) KcsA-WT (C) KcsA-R64A and (D) KcsA-R64D. Increasing concentrations of TFE are represented by decreasing color intensity of the lines. All experiments were performed at 5 mM KCl and pH 7.4. (E) Emission maximum wavelength of tryptophan- fluorescence of KcsA-WT, KcsA-R64A and KcsA-R64D as function of vol% TFE. Again as in figure (A) KcsA-WT is represented by a solid line, KcsA-R64A by a dashed line and KcsA-R64D by a dotted line.

Lipid dependence of tetramer stability in KcsA-R64 mutants

Next we investigated the lipid dependency of the tetramer stability of the two KcsA-R64 mutants as compared to KcsA-WT. For this we performed a TFE titration on the WT protein and both mutants, reconstituted in different lipid vesicles and we followed the amount of tetramer by SDS-PAGE. *Figure 4* shows that KcsA-R64D has a strongly increased tetramer stability in all bilayers, except in PC/PE *(Fig 4. D*). In contrast, KcsA-R64A has similar tetramer stability as KcsA-WT in all lipid bilayers tested, except in PC *(Fig 4. A),* where the stability is significantly increased. The general stabilization effect in KcsA-R64D might be due to an electrostatic bond between R64 and R89 of adjacent monomers, which is formed in binding pockets that are not occupied by a lipid. The possible reasons for the lipid specific effects will be discussed later.

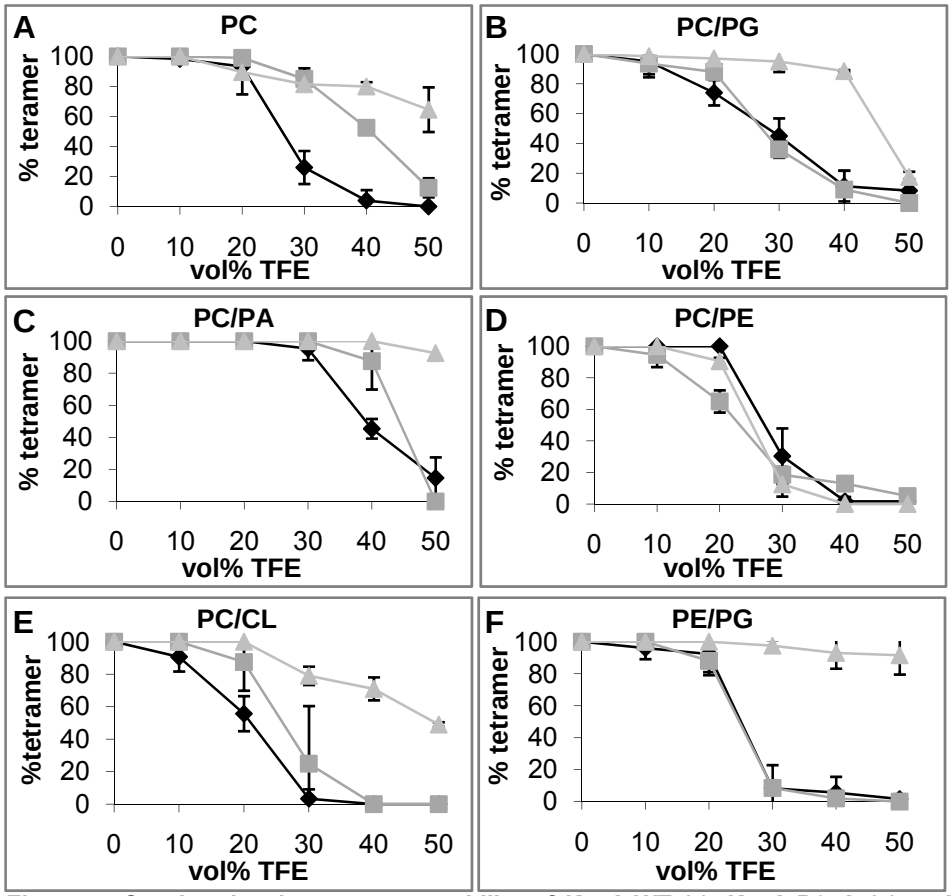

Figure 4: Graphs showing tetramer stability of KcsA-WT (♦), KcsA-R64A (■) and KcsA-R64D (▲) as function of TFE concentration in different lipid bilayers. (A) PC, (B) PC/PG, (C) PC/PA, (D) PC/PE, (E) PC/CL and (F) PE/PG. For all mixed bilayers the molar ratio of the indicated lipids was 7:3. All data points represent an average of three independent experiments. The error bars represent the standard deviation. All experiments were performed in a buffer containing 5 mM KCl and at pH 7.

Allosteric effect of lipid binding

Since residue R64 in KcsA-WT seems to be involved in lipid binding as well as in tetramer stability it would be interesting to identify lipids that are bound to the lipid binding pocket in KcsA-WT as well as the mutants. In previous mass spectrometry studies it was found that PG binds preferentially to KcsA over PE and PC [17]. However, the results obtained in this previous study can not directly be correlated to the presence of lipids in the binding pockets, because KcsA was dissociated into its monomeric form by the addition of TFE, resulting in alteration or removal of the specific binding pocket. Here we used a recently developed alternative approach [16] to directly analyze native KcsA-WT purified from *E.coli* membranes and solubilized in DDM micelles by mass spectrometry, thereby minimizing alterations in lipid occupation of the binding sites.

These experiments on native KcsA in DDM made it possible to visualize KcsA-WT in its tetrameric form by mass spectrometry for the first time. *Figure 5A* shows clear peaks with charges states 16^+, 17^+ and 18^+ representing the KcsA tetramer. The average mass of the tetramer found at these charge states is 78497 Da with a deviation of $^+/_-$ 6 Da. This indicates that besides the 77097.7 kDa tetramer (4 times the theoretical value of the monomer 19274.43 [17]) most likely also two lipids are present to account for the mass difference of approximately 1400 Da. Unfortunately is was not possible to identify the nature of any bound lipids. However the MS experiments did allow us to further investigate the noncovalent interactions of this tetrameric protein. It was expected that upon entering the collision cell the KcsA-WT tetramer would fragment into monomeric subunits. However MS/MS experiments performed on the 17^+ ions at m/z 4610 *(Fig. 5A)* show that the KcsA-WT tetramer dissociates into dimers, indicated with a D in *Fig. 5B.* This unusual dissociation behavior indicates that within the KcsA-WT tetramer the monomers forming a dimer are bound together in a way that solidifies their interaction, over the interaction between all monomers. In this experiment the average total mass of the dimer, based on the peaks with charge state 5^+, 6^+ 11^+ and 12^+, is 39235 $^+/_-$ 15 Da, which is higher compared to the theoretical value of the dimer (38548 Da [17]). The additional mass of around 700 Da per dimer, could very well represent a lipid which is bound to the lipid binding pocket between two monomers.

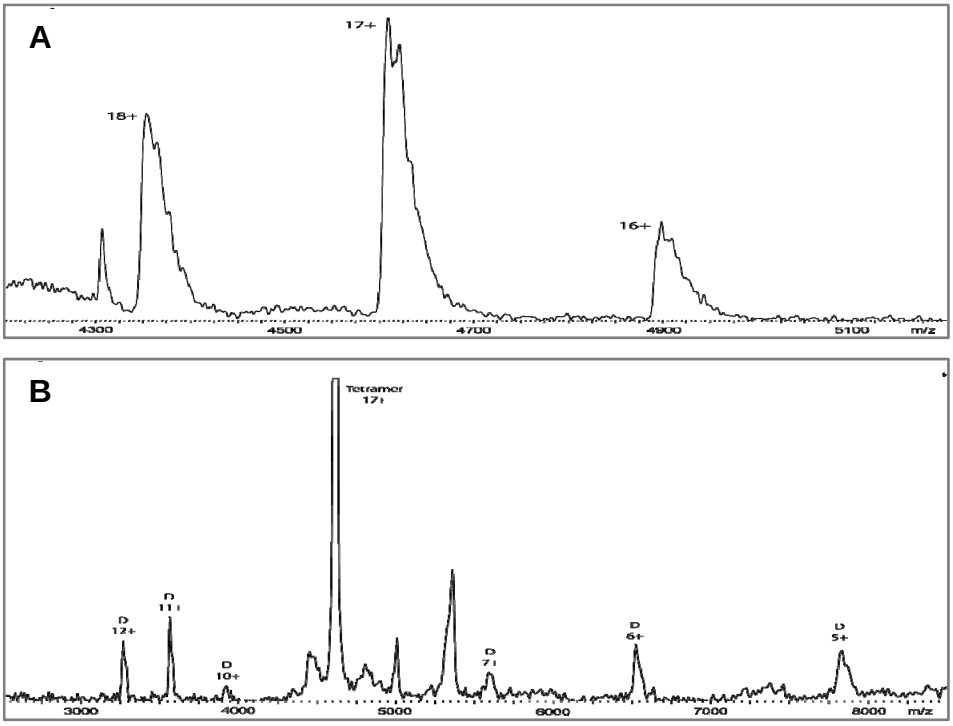

Figure 5. Mass spectrum of native KcsA-WT showing, (A) the tetramer peaks of KcsA-WT as 16^+, 17^+ and 18^+ ions. (B) MS/MS spectrum of the 17+ ion from the tetramer spectrum, showing clear high and low charged dimer peaks, all indicated with Dx^+ with x representing the charge state.

Similar experiments as described above were attempted for both KcsA-R64 mutants to determine if indeed these mutants have altered lipid binding properties or whether they show differences in tetramer or dimer stability. However to date these experiments were not successful.

Discussion

Previously, it was found that lipids influence KcsA tetramer stability [2-4]. Moreover a specific interaction for PA with the KcsA-WT tetramer was suggested [13], which was further investigated and established in the previous chapter. The results showed that PA has a specific interaction, depending on charge, with the KcsA-tetramer thereby increasing its tetramer stability. Additionally it was found that PA did not have this stabilizing effect on the chimeric channel KcsA-Kv1.3 tetramer, indicating that the interaction site of PA with the KcsA-Kv1.3 tetramer lies within the residues that are altered as compared to KcsA-WT. Together with the location of the lipids that were found in the KcsA-WT crystal structure [10] and the model that indicates that R64 and R89 are the key players in lipid binding, with a preference for PA [9], this motivated us to alter R64 into an alanine or aspartic acid in order to investigate the exact role of R64 in lipid binding.

The results of the present study shows that residue R64 in the potassium channel KcsA indeed is important for tetramer stability. Below the proposed mechanism of involvement of R64 in tetramer stabilization and thereby lipid binding will be discussed in more detail.

Influence of R64 mutations on tetramer stability

The *in vivo* TFE titration followed by SDS-PAGE to visualize the tetramer showed that especially KcsA-R64D has a huge enhancement in tetramer stability compared to KcsA-WT. This increase of stability resembles the increase that is observed in the presence of PA for KcsA-WT, suggesting that in KcsA-R64D PA binding somehow is mimicked. Moreover it demonstrates that the impaired stability in KcsA-Kv1.3, as found in chapter 3 is not simply due to the R64D mutation, but that other alterations compared to KcsA-WT must play a role in the compromised stability in the chimera. The fact that KcsA-Kv1.3 has four negative charges in the eleven amino acids that are altered compared to two positive charges in KcsA-WT might be responsible for this lower stability. It is possible that these negative charges rearrange the protein structure in such a way that the lipid binding pocket is altered or does no longer exist as such thereby prohibiting specific lipid binding. Despite extensive research on KcsA-Kv1.3 in relation to toxin binding, gating [18-20] and structural studies [21-23], the only data obtained on tetramer stability of this chimera is that which is discussed in the previous chapter, showing that the KcsA-Kv1.3 tetramer is indeed not stabilized by PA as was found for KcsA-WT.

Residue R64 and R89 have been modeled to form the main lipid binding pocket for specific lipids [9], as illustrated in *Fig. 6A,B* and *C* [24, 25]. It is likely that upon PA binding these flexible side chains can position themselves with respect to one another in such a way that the positive charges can optimally interact with the negative charges on the PA head group, and that these electrostatic interactions are enhanced by the specific charge properties of PA, as discussed in chapter 3. Indeed, the modeling study [9] suggests that

PA has a slightly stronger affinity to this pocket than PG and much stronger than PE, in accordance with our (chapter 3) and previous findings [13] that PA stabilizes the KcsA-WT tetramer in a more significant way compared to other lipids. However this finding does not rule out that other lipids can bind to the same binding pocket, especially since the amount of PA in bacterial membranes is very low compared to PG and PE [26]. Mass spectrometry experiments show that the KcsA-WT tetramer dissociates into two dimers, rather than a trimer and monomer. This stabilization effect could very well be due to the presence of (different) lipids in the specific binding pockets. Thus, binding of one lipid to the monomer-monomer interface of two subunits might stabilize the dimer. In addition, such binding could affect the lipid binding site on the adjacent monomer via an allosteric effect, thereby changing its lipid affinity and/or specificity. A tight regulation of lipid binding could be important for channel functioning, as for example supported by fluorescence quenching studies that indicate that at least three of the lipid binding pockets should be occupied to enable gating [15], and hence that the channel remains closed if less than three lipids are bound.

The stabilization of dimers as observed by MS/MS experiments supports the idea of an allosteric effect of lipid binding, since in the absence of such an effect all monomer-monomer interactions would be similar, causing monomers to dissociate from the tetramer rather than splitting up in dimers. To gain more insight into the molecular nature of these effects it would be important to identify the lipids bound to these dimers and to characterize any structural changes of the proteins upon binding of different lipids.

Model for increased stability in KcsA-R64D

Replacing the positive charge of R64 with the negative charge of aspartic acid may have several implications for the lipid binding properties of the pocket. First of all the hydrogen bond that is modeled to form between the lipid and R64 can not be established any more [9], making R89 the key player in lipid binding. The change in potential binding space as well as binding affinity upon substitution of R64 could cause a wider variety of lipids to bind to the pocket compared to KcsA-WT thereby generating a wide range of possibilities to influence tetramer stability and thereby possibly channel function. Another major consequence of replacing R64 by an aspartic acid and thereby changing the charge is that it opens up the possibility of direct electrostatic interactions between D64 and R89, as illustrated in *Fig. 6D.* Such a direct interaction could explain why R64D is much more stable compared to KcsA-WT and KcsA-R64A. This interaction however can only be formed in the absence of lipids in the binding pocket, since the two residues must be in close enough proximity and free to interact with each other. It is possible that upon assembly of KcsA-WT or its mutants, lipids are incorporated in the binding pockets of the tetramers.

However, it has been suggested that such lipid binding is transient in order to enable gating [15]. Hence, when these bound lipids in KcsA-R64D “leave” the binding pocket, this enables D64 and R89 to interact with each other. The presence of such a direct interaction is consistent with the tryptophan fluorescence results that show a major red shift for KcsA-R64D between 30 and 50 vol% TFE without dissociation of the tetramer into monomers. This indicates that the tetramer is undergoing significant structural rearrangements, moving

the tryptophans into a more hydrophilic environment without being able to dissociate into monomers. This behavior is not observed for KcsA-WT and KcsA-R64A and we suggest that the bond that keeps the monomers together in KcsA-R64D is a direct, electrostatic interaction between D64 and R89.

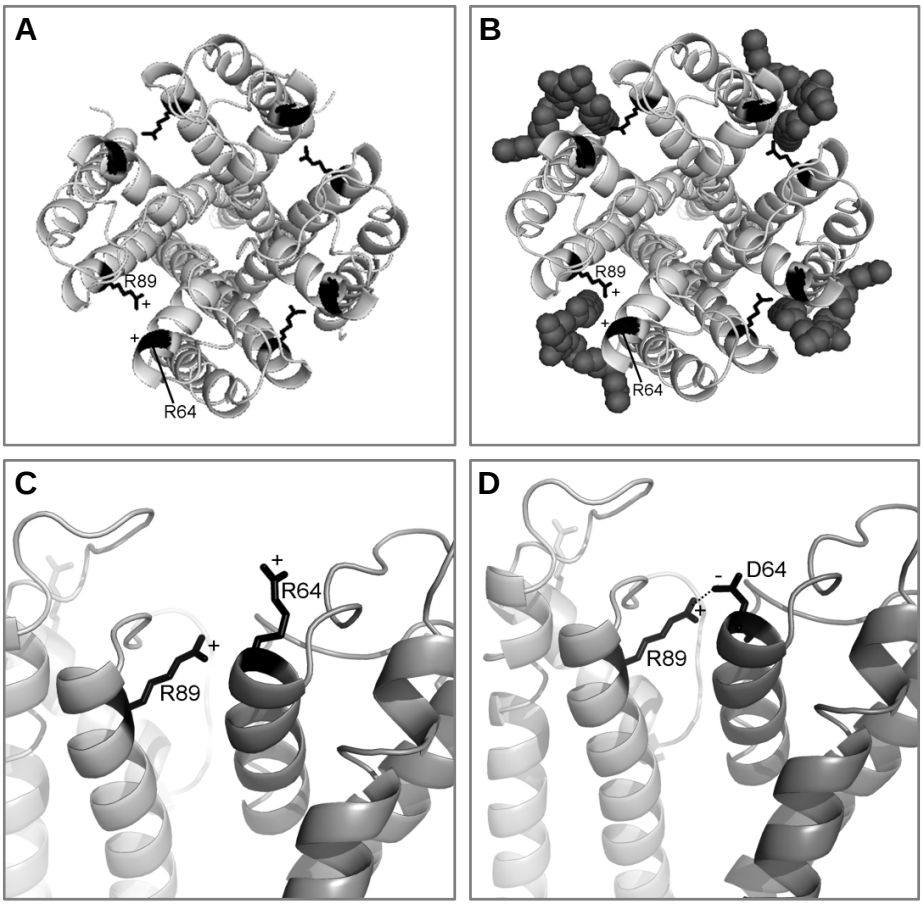

Figure 6: Model for enhanced tetramer stability. (A) and (B) Cartoons showing the top view of KcsA-WT in which the assigned, black, residues R64 and R89 are the key players in lipid binding (A) KcsA-WT without lipids bound. (B) KcsA-WT with diacylglycerol modeled to be bound to the lipid binding pockets based on PDF 1k4C published by Zhou et al. 2001. (C) Close up cartoon of the lipid binding pocket of KcsA-WT highlighting residue R64 from one, and R89 from the adjacent monomer as the main residues involved in lipid binding. (D) Close up cartoon of the lipid binding pocket in KcsA-R64, indicating the possibility of a direct interaction between D64 of one, and R89 of the adjacent monomer with a dashed line. For clarity in figures (C) and (D) the left monomer is represented in light grey whereas the right monomer is shown in dark grey. All cartoons are based on PDB 3EFF as published by Uysal et al 2009 .

Lipid dependence of tetramer stability in KcsA-R64 mutants

In general the tetramer stability of KcsA-R64D is much larger than that of KcsA-R64A, which is comparable to that of KcsA-WT. The observation that the increased stability in KcsA-R64D is not present in PC/PE might be due to the fact that PE, because of its relatively small head group and its positively charged aminogroup is still able to bind to the lipid binding pocket via electrostatic interactions with D64 as well as R89, thereby prohibiting the direct interaction between D64 and R89 and consequently facilitating a drop in stability. Why then would this binding of PE be overruled in the presence of PG as can be seen in the PE/PG graph *(Fig. 4F)*. We speculate that in PE/PG mixtures the partitioning of PE to the lipid binding pocket is less favorable than in PC/PE mixtures, because PE and PG have much better mixing properties in a lipid bilayer than PC and PE [27].

For KcsA-R64A it was shown that in DDM, pure PC and *in vivo* it shows a slight increase in tetramer stability as compared to KcsA-WT. A possible explanation is that in these bilayers, besides the favorable anionic lipids, other lipids and/or detergents can bind better because the substitution of arginine to the smaller alanine results in a larger available space within the binding pocket. In the other lipid mixtures the advantage of increased space in this mutant then may be counteracted by the advantage in KcsA-WT that interaction with anionic lipid is more favorable.

Possible consequences of R64 mutations for channel properties

As described above the R64 mutations in KcsA most likely alter the lipid binding properties of these mutants as compared to KcsA-WT. Residue R89 in KcsA-WT, a proposed partner of R64 in the lipid binding pocket, has been found to be essential for channel functioning [9, 10]. Alterations in residue R64 could therefore also be of influence on the functional properties of the channel. For instance, the binding of zwitterionic PC to the binding pockets of KcsA-R64A could mimic the binding of anionic lipids, which are necessary for channel opening. Consequently more channels may obtain the open conformation, thereby causing an increase in tetramer stability in this environment. This would be in agreement with the increased open probability that has been found for KcsA-R64A in asolectin vesicles [28]. Similarly the formation of the salt bridge between D64 and R89 in KcsA-R64D could cause the channels to open. However, channel properties of this mutant have not yet been analyzed. Thus, functional studies on these mutants can be expected to yield important new insights into the molecular nature of lipid binding to these specific binding pockets, especially when combined with further experiments on both KcsA-R64 mutants to analyze the nature of any bound lipids or the tetramer stability, for example by fluorescence methods or by mass spectrometry as in the present study.

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Chapter 5

Functional properties of KcsA-R64 mutants

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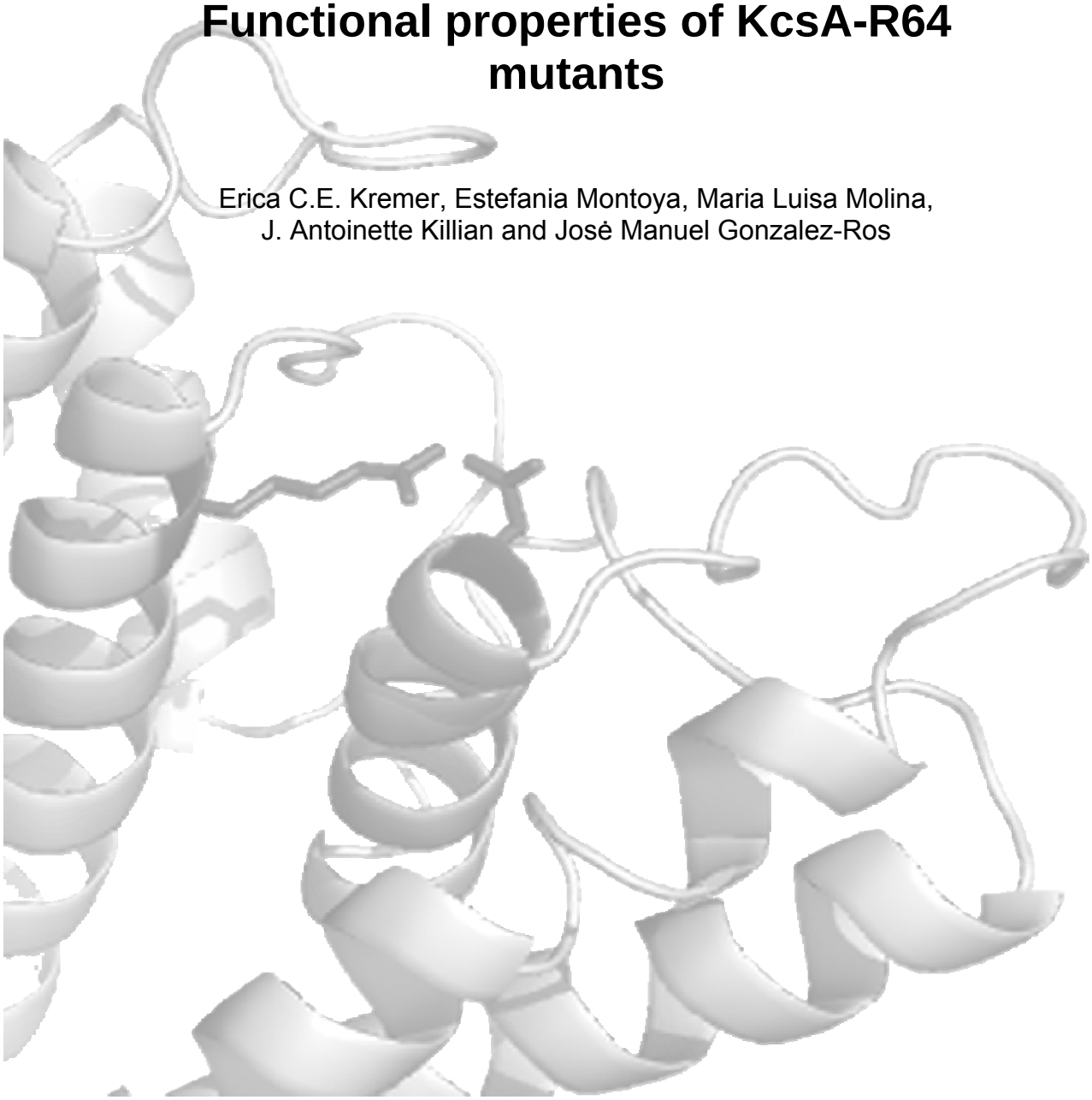
J. Antoinette Killian and Josė Manuel Gonzalez-Ros



Abstract

The structural and functional properties of the potassium channel KcsA from the bacterium *Streptomyces lividans* are known to be influenced by lipids in various ways. Residue R64 in KcsA is implicated to be part of the lipid binding pocket and is involved in tetramer stability *(chapter 4).* In this study we investigated whether R64 is also involved in channel functioning. For this purpose we analyzed the channel conductance of two KcsA mutants (KcsA-R64D and KcsA-R64A) which were reconstituted into giant asolectin vesicles. It was found that both mutants had a similar conductance as KcsA-WT including the ability for high and low open probability gating. However both mutant channels were found to be less stable compared to KcsA-WT: the majority of KcsA-WT channels gate during periods of time up to hours, whereas a large number of mutant channels deactivates shortly after stimulation. This indicates that R64 plays a role in conformational rearrangements that are necessary for channel gating. Together the results suggest that gating and tetramer stability are linked, and that lipid binding to R64 is an key feature for both.

Introduction

One of the best understood ion channels is the potassium channel KcsA from the bacterium *Streptomyces lividans*. Although KcsA has been investigated extensively, the exact mechanism of gating remains unclear. The general consensus is that KcsA most likely contains two gates: one at the selectivity filter and one close to the C-terminal domain. The gate at the selectivity filter has a necessity for bound potassium to remain in the open state [1, 2], it is voltage dependent [2, 3], and its open probability can be increased by mutations in and near the selectivity filter [4-6]. The gate at the C-terminal domain is responsible for the pH sensitivity of the channel. KcsA is found to open at low pH [7, 8] and the pH sensor is most likely formed by residues which are located in or in close proximity of the C-terminal domain [6, 8-11]. The gates at the selectivity filter and at the C-terminal domain are most likely coupled, governing coordinated sequential opening [8, 11-13].

KcsA has a diverse functional behavior. The most commonly observed patterns of activity correspond to the low open probability (LOP) pattern, which is an acidic pH-dependent state, and the high open probability (HOP) pattern, which is present at acidic as well as neutral pH. This latter state shows frequent coupled gating of multiple channels [14]. Furthermore it was found that KcsA is only active in the presence of anionic lipids [15]. This necessity for anionic lipids could follow from general electrostatic interactions between the KcsA tetramer and the lipid bilayer, but it could also be that lipid binding at a specific site is required. The latter possibility seems likely since tightly bound lipids were found at the interface between adjacent monomers in the KcsA crystal structure [16]. The binding site for these lipids was modeled to mainly consist of R64 of one monomer and R89 of the adjacent monomer [17], which would explain the high affinity for anionic lipids. It was found that at least three of these lipid binding sites need to be occupied by anionic lipids in order to allow opening and to facilitate gating [18]. This implies that possibly the binding of (certain) lipids to

this cleft might play a role in the activation and inactivation of the selectivity filter.

Previously we found that mutating R64 into an aspartic acid results in an increased tetramer stability in virtually all lipid bilayers that were tested. This suggested that D64 an R89 can form an intermolecular salt bridge that inhibits lipid binding, but that nevertheless strengthens interactions between the subunits, thereby stabilizing the tetramer. In the present study we performed functional assays on KcsA-WT and KcsA-R64D to gain more insight in the involvement of residue R64 in gating of KcsA. As a control we also studied the functional properties of the mutant KcsA-R64A, of which the group of Perozo has shown that it has similar gating behavior as KcsA-WT, but with a somewhat increased open probability [19]. We show that also KcsA-R64D is able to gate in a similar way as KcsA-WT. However both KcsA-R64 mutants are less stable since a large percentage of these channels tends to remain closed after some time, implying that R64 plays a role in KcsA gating. We postulate that lipids might form a key feature in this process. The role of lipids in gating and the possible relation between gating and tetramer stability will be discussed.

Materials and Methods

Materials

The following chemical reagents were purchased either from Fluka, Merck or Sigma Aldrich, available in the highest purity: Lysozyme, HEPES, KCl, NaCl, imidazole and isopropyl-β-D-thiogalactopyranoside (IPTG). Asolectin (soybean lipids type II-S) was purchased at Sigma Aldrich, n-Dodecyl-β-D-maltoside (DDM) was obtained from Anatrace Inc, Ni^{2+}-NTA agarose from Qiagen, Merck and Coomassie Brilliant Blue G-250 from ICN Biomedicals. Furthermore the electrophoresis setup, proteins markers and Bio-Beads SM-2 adsorbent were purchased at Biorad laboratories.

KcsA mutagenesis

KcsA point mutants were obtained via quickchange mutagenesis kit from Stratagene. Primer design and PCR were optimized by following the supplied protocol. Pt7-837KcsA was used as template DNA. Obtained mutations were verified by DNA sequencing.

Protein expression and purification

KcsA mutants were expressed as previously described in [20]. Briefly, proteins were expressed with an N-terminal His-tag from pT7-KcsA in *Escherichia coli* strain BL21 (λDE3) upon IPTG induction [20]. KcsA was purified in a buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM KCl. The membranes were solubilized in 3 mM DDM and incubated with pre-washed Ni^{2+}-NTA agarose beads overnight at 4°C. The bound His-tagged proteins were eluted with 300 mM imidazole pH 7.5 and 1 mM DDM. The purity of the proteins was assessed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The presence of DDM precluded the use of the Bradford assay, and therefore the protein concentration was assessed by SDS

gel after staining with Coomassie Blue, using a standard of bovine serum albumin (BSA).

KcsA-WT was expressed as described in Molina et al (2004 and 2006) [14, 21].

SDS-PAGE stability assay

This assay was performed as previously described in [22]. Briefly, membrane fractions of cells overexpressing KcsA, or purified protein in DDM or vesicles were divided in equal aliquots of 10 µl containing approximately 2,5 µg of protein and incubated with increasing amounts of TFE for one hour at room temperature. TFE was added in an aqueous solution with a total concentration of 5 mM KCl, 45 mM NaCl to minimize changes in ionic strength. The TFE containing samples, with a total volume of 13 µl, were mixed with 5 µl of an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8, 50% glycerol, 0.01% bromophenol blue and 10% SDS) and directly run on 15% acrylamide gel in the presence of 0.1% SDS at room temperature. Afterwards proteins were visualized by Coomassie Brilliant Blue staining. The gels containing purified protein samples were scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The tetramer band intensity present at 0 vol% TFE was set to 100%. The amount of tetramer (%) was plotted against the TFE concentration (vol%) for the stability assay.

Vesicle preparation and protein reconstitution

Large unilamellar vesicles (LUVs) were prepared by extrusion through 200nm filters [22]. LUV's (5 mM phospholipids) were prepared in vesicle buffer (10mM HEPES, pH 7.5, 45mM NaCl, 5mM KCl) solubilized with 1% Triton X-100 (w/v) and mixed with DDM solubilized KcsA mutant proteins at a 1:2000 protein: lipid molar ratio. The detergent was removed using pre-washed Bio-Beads. The reconstituted vesicles were collected by centrifugation (1 hour, TLA 100.1 rotor, 100000 rpm at 4°C). The proteoliposomes were finally resuspended in one of the buffers described above.

KcsA-WT was reconstituted as described in Molina et al (2006) [14].

Preparation of giant liposomes

Batches of large unilamellar vesicles of asolectin (soybean lipids, type II-S, Sigma) were prepared at 25 mg/ml as described earlier [23] in 10 mM Hepes, pH 7.0, 100 mM KCl (reconstitution buffer) and stored in liquid N_2. Multilamellar giant liposomes (up to 50–100 µm in diameter) were prepared by submitting a mixture of the reconstituted vesicles and asolectin lipid vesicles (25 mg of total lipids) to a cycle of partial dehydration/rehydration [23], with the exception that the dehydration solution used here was 10 mM Hepes (potassiumsalt) buffer, pH 7, containing 5% ethylene glycol and the rehydration solution was 10 mM Hepes (potassium salt) buffer, pH 7. As a control, each of the different batches of asolectin vesicles was also used to prepare protein-free giant liposomes. Liposome batches were discarded when they posed difficulties to obtain high resistance seals or when they showed erratic baselines in the patch clamp recordings, either because of remaining detergent or because of other, unknown reasons.

Electrophysiological recordings

For patch clamp measurements of channel activity, aliquots (2-3µl) of giant liposomes were deposited onto 3.5-cm Petri dishes and mixed with 2 ml of the buffer of choice for electrical recording (bath solution; usually 10 mM Mes buffer, pH 4,containing 100 mM KCl). Giga seals were formed on giant liposomes with borosilicate microelectrodes (Sutter Instruments) of 8-16 MΩ open resistances, filled with 10mM Hepes buffer, pH 7, 100mM KCl (pipette solution). After sealing, excised inside-out patches were obtained by withdrawing the pipette from the liposome surface. Standard patch clamp recordings [24] were obtained using EPC (Heka Electronic, Lambrecht/Pfalzt, Germany) patch clamp amplifiers, at a gain of 50mV/pA. The holding potential was applied to the interior of the patch pipette, and the bath was maintained at virtual ground ($V = V$ bath $+V$ pipette). An Ag-AgCl wire was used as the reference electrode through an agar bridge, and the junction potential was compensated when necessary. Routinely, the membrane patches were subjected to a protocol of pulses and/or voltage ramps. The protocol of pulses went from -200 to +200 mV, at 50 mV intervals, and 2 seconds of recording at each individual voltage was used, holding the patch back to 0 mV between the different voltage steps. Additionally 30 second recordings at selected voltages were made for some patches. The voltage ramps went from -200 to +200 mV, during a 3 s scan. All measurements were made at room temperature. Recordings were filtered at 1 kHz

Results

Tetramer stability of KcsA reconstituted in asolectin

Previous TFE titration experiments in lipid bilayers showed that KcsA-R64D has an increased tetramer stability compared to KcsA-WT, while KcsA-R64A has a similar stability. These experiments were performed in different types of bilayers, mostly representing binary mixtures of lipids (*chapter 4*). However, these systems are less suitable for functional studies, which sofar have been performed mainly in asolectin. Also in the present study we made use of this convenient, but poorly defined lipid mixture. We first tested whether KcsA-WT, KcsA-R64A and KcsA-R64D behave similarly in asolectin as in the other lipid systems tested with respect to tetramer stability. *Fig. 1A* shows that also in asolectin KcsA-R64D has increased tetramer stability whereas for KcsA-R64A a similar stability compared to KcsA-WT is found.

A remarkable feature observed is that KcsA-R64A, KcsA-R64D as well as KcsA-WT show an extra protein band above the 64kD tetramer band on SDS-PAGE, as illustrated for KcsA-R64D in *Fig. 1B*. Previously it was suggested by Barrera et al. [25] that the extra band represents KcsA tetramers forming a stable complex with other bacterial components [26]. However, this is unlikely because this band thus far has never been observed by us in any of the other synthetic lipid mixtures or *in vivo*, indicating that it may be specific for asolectin. Thus, an alternative explanation might be that asolectin induces a structural rearrangement in the tetramer leading to aberrant behaviour on the gel, or alternatively, that it induces cluster formation.

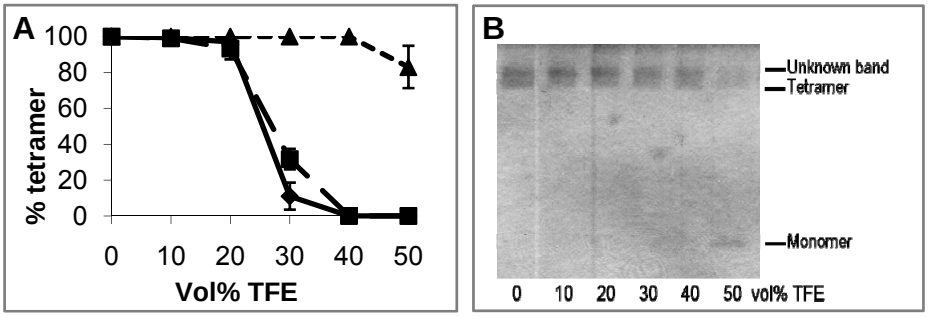


Figure 1: Tetramer stability in asolectin. (A) Graph showing percentage of tetramer as function of concentration of TFE present for KcsA-WT (solid line, ♦), KcsA-R64A (dashed line, ■) and KcsA-R64D (dotted line, ▲). (B) Representative 15% acrylamide gel for KcsA in asolectin. In this gel the tetramer stability of KcsA-R64D is shown upon TFE titration. The tetramer and unknown band are present from 0-40 vol% TFE, whereas at 50 vol% TFE these bands disappear and dissociate into monomers.

Functional behavior of KcsA-R64 mutants

To investigate the functional properties of the two R64 mutant channels first a continuous recording of the channels activity was taken at -150 and +150 mV. *Fig. 2* shows a conductive pattern for all three proteins in which most channels are active during the entire measurement. For KcsA-WT the conductance of a single channel under these conditions is approximately 4 pA. The fact that both at positive and negative voltage the current is relatively constant and manyfold higher than 4 pA (*Fig. 2 A and D),* implies that many channels are open at the same time and that they remain open together as cluster. The spikes on these lines represent bursts of activity corresponding to rapid opening and closing of single channels. At about 20 sec a dip of approximately 20 pA is present in the conductivity in *Fig. 2A*, corresponding to the simultaneous closing of several channels to reopen a few seconds later. Together these results indicate that the channels are coupled in their gating. Similar patterns are found for both KcsA-R64 mutants (*Fig. 2, B+E, C+F).* When taking a close look at these registers, it was found that all three proteins show a basic current of approximately 4 pA per channel with a frequent occurrence of coupled gating of two or more channels (data not shown). Additionally it was found (data not shown) that both KcsA-R64 mutants are able to have a “low channel opening probability” (LOP) as well as a “high channel open probability” (HOP) as previously described for KcsA-WT [14]. LOP patterns are generally characterized by recordings showing a few openings in the form of bursts, while the channels are closed most of the time. In the HOP pattern the channels are opened most of the time and characteristically channel closings are observed at extreme voltages whereas variable flickering can occur at any of the voltages studied. In some HOP patterns more current is conducted during a negative potential compared to positive potential, as also observed in *Fig. 2* for KcsA as well as for both mutants. In an I-V measurement the behavior of the channels results in a sigmoid-like I-V curve. In KcsA-WT both LOP and HOP patterns are

observed in 45% of all patches, whereas the remainder 10% shows intermediate behavior or is silent [14]. All continuous recordings shown in *Fig. 2* show a HOP pattern. The occurrence of both HOP and LOP patterns implies that the basic gating behavior of both KcsA-R64 mutants is similar to that of KcsA-WT.

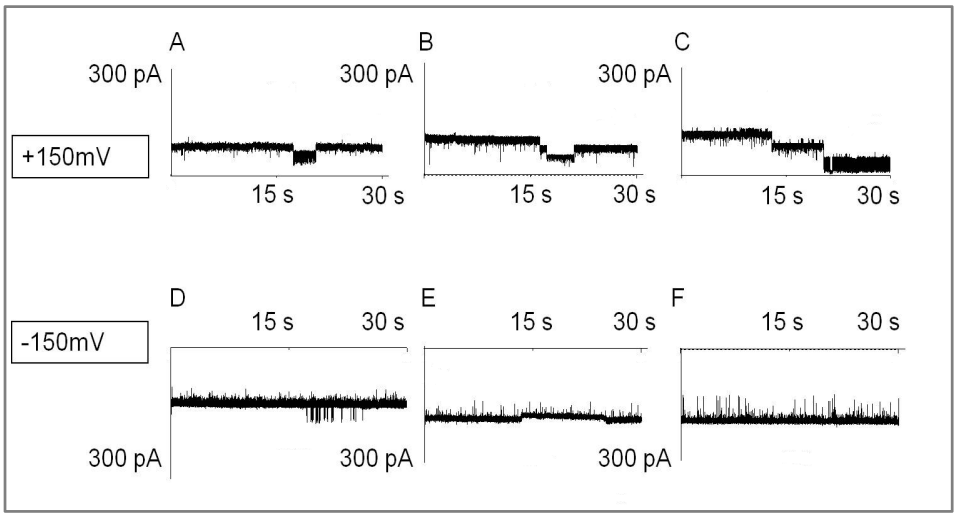


Figure 2: Functional recordings of KcsA-R64 mutants. The gating behavior of KcsA-WT (A+D), KcsA-R64A (B+E) and KcsA-R64D (C+F) reconstituted in asolectin is shown upon a continuous pulse of 30 sec at +150 mV (A-C) and -150 mV (D-F). The Y-axis represents the total current of the present channels and ranges from 0-300 pA.

Functional stability of KcsA-R64 mutants

Apart from the similarities in channel properties, also a striking difference was observed between KcsA-WT and both mutants. While KcsA-WT channels normally remain active till the channels are chemically blocked by e.g. addition of NaCl [27, 28] or Shaker-B peptide [29, 30], a percentage of the KcsA-R64 mutants, which is higher compared to KcsA-WT, has the tendency to close by themselves. *Fig. 3A* shows representative successive I-V ramps measured between -200mV and +200 mV for KcsA-WT. These ramps show a HOP pattern that remains stable for thirteen minutes, but similar ramps could be observed till well over an hour. On the other hand, *Fig. 3B* shows a HOP pattern for KcsA-R64A which closes within ten minutes, whereas *Fig. 3C* in this particular case shows a typical LOP pattern for KcsA-R64D at t=0 which also closes rapidly.

The reduced stability is representative for both mutant channels. In KcsA-R64A the increase in the number of patches that deactivates is relatively small: 37% compared to 20% for KcsA-WT. In KcsA-R64D however the percentage of patches that deactivate themselves is around 85%. Additionally the estimated rate of deactivation seems to be similar for all three channels.

This suggests that only the percentage of patches that deactivates increases, whereas the rate of deactivation for a given patch remains fairly constant.

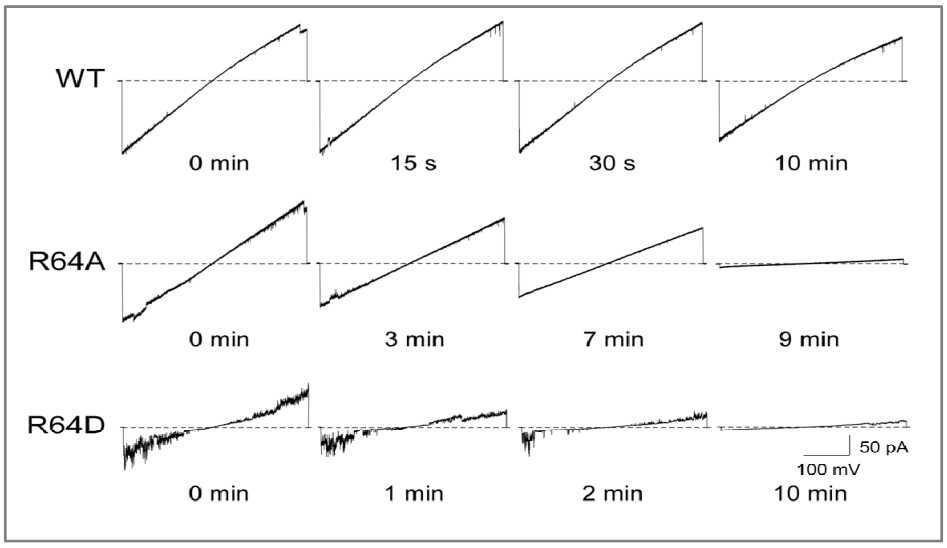


Figure 3: Recordings of successive I-V ramps measured between -200 mV and +200 mV (x-axis) are shown for (A) KcsA-WT, (B) KcsA- R64A and (C)KcsA- R64D. The current is depicted on the y-axis and ranges from -200 pA till +200 pA. Each curve intersects the axes at 0 pA and 0 mV. The time, depicted under each graph, represents the number of seconds/minutes that have passed since the initial pulse.

Influence of external blockers on KcsA-R64 mutants

The presence of a constitutively active population of channels, albeit small, gave us the opportunity to perform an additional, but sofar preliminary, functional test on the KcsA-R64 mutant channels. For KcsA-WT it is known that the addition of 5 mM NaCl can silence the LOP pattern [28] while the HOP pattern is insensitive to NaCl addition [14]. Our experiments suggest a similar behavior for KcsA-R64D. *Fig. 4* shows successive ramps of KcsA-R64D showing a transition from a HOP to a LOP at 30 minutes, similar as can be observed for KcsA-WT [14]. The figure also shows that at 40 minutes and upon addition of 5 mM NaCl no current is observed anymore for KcsA-R64D, suggesting that also here the LOP pattern can be silenced by NaCl. However, in our study, not sufficient experiments have been performed to rule out that the closing of channels at 40 minutes is unrelated to the addition of NaCl. In contrast, the HOP pattern of KcsA-R64A like that of KcsA-WT was not silenced upon addition of NaCl till a concentration of 20 mM, indicating a similar mechanism of interaction *(data not shown)*.

These results are in agreement with observations made by the group of Perozo [19] and indicate that both R64 mutations do not influence the channel silencing by NaCl. However it has to be noted that these results are obtained from only a few experiments and that it could very well be that small differences in sensitivity towards NaCl do exist.

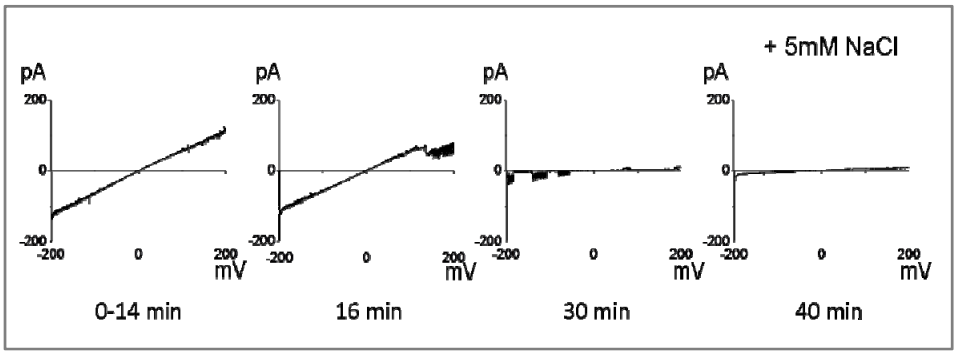


Figure 4: Recordings of successive I-V ramps for KcsA-R64D measured between -200mV and +200 mV (x-axis) in the exceptional situation that the channel remains open after 10 min. The current is depicted on the y-axis and ranges from -200 pA till +200 pA. Each curve intersects the axes at 0 pA and 0 mV. The time that has passed since the initial pulse in indicated below each graph in minutes. After 40 min 5 mM NaCl is added which chemically closes the channel. The time depicted under each graph represents the number of minutes that have passed since the initial pulse.

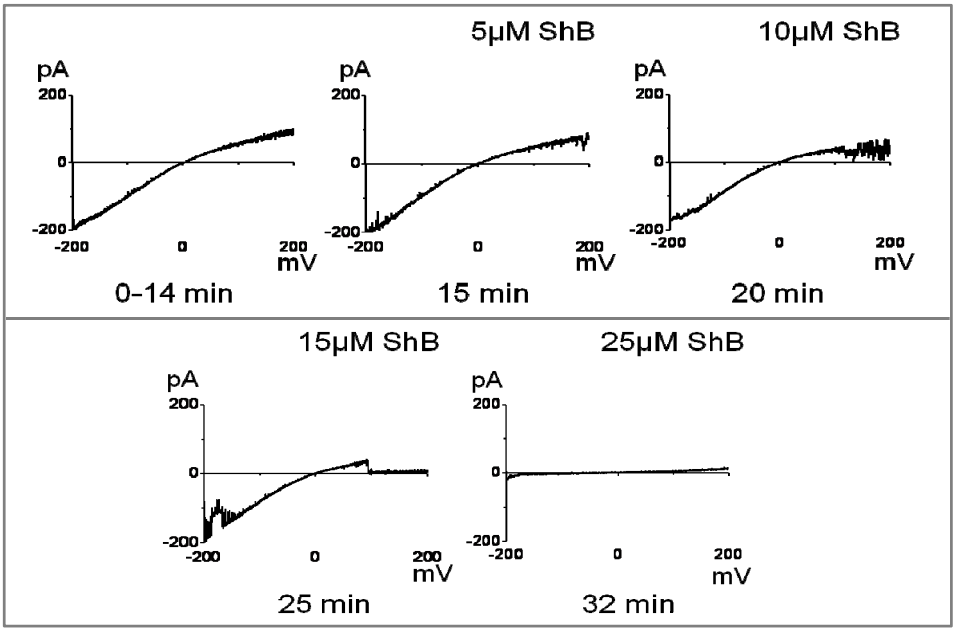


Figure 5: Five recordings of successive ramps for KcsA-R64A measured between -200mV and +200 mV (x-axis) in the exceptional situation that the channel remains open after 10 min. The current is depicted on the y-axis and ranges from -200 pA till +200 pA. Each curve intersects the axes at 0 pA and 0 mV. The concentration Shaker B-peptide is indicated in µM above the graphs. The time depicted under each graph represents the number of minutes that have passed since the initial pulse.

Besides NaCl we also made use of the Shaker B peptide to investigate possible differences in function compared to KcsA-WT. In KcsA-WT the Shaker B peptide can silence both LOP and HOP patterns. We found that also in the KcsA-R64A both gating patterns can be blocked by this peptide. *Fig. 5* shows a HOP pattern in KcsA-R64A that is gradually silenced by the addition of increasing concentrations of shaker B peptide. Similar to the NaCl blockage it seems that the general blocking features of the Shaker B peptide are not compromised by the R64 mutation. However, also here subtle differences in sensitivity towards the peptide could surface when more experiments are performed. At the present it seems that the R64 mutation does not disturb inactivation of the KcsA channels by the Shaker peptide.

Discussion

The results indicate that the KcsA-R64D and the KcsA-R64A mutants both have similar gating features as compared to KcsA-WT. However the fact that a larger percentage of the mutant channels closes irreversibly within a short period of time after stimulation indicates that R64 must be involved in the stability of the open conformation of the channels. Below we will present a model to explain the molecular mechanism of this impaired stability in gating and next we will discuss other features found for the KcsA-R64 mutants in the described experiments.

Functional behavior of KcsA-R64 mutants

It was found that KcsA-R64A as well as KcsA-R64D have different types of activity within identical experiments. These activities have also been found for KcsA-WT and they are characterized as HOP and LOP patterns. Since their occurrence can not be related to experimental variables, they must represent the true behaviour of the channels themselves [14]. The occurrence of HOP patterns indicate that both mutants channels, like KcsA-WT can be functionally coupled.

Altered distributions of HOP vs. LOP activities were found in both KcsA-R64 mutants. It seems that KcsA-R64A has a slightly larger percentage of HOP patterns compared to KcsA-WT (45%) [14], whereas KcsA-R64D only had a HOP in 13% of the recordings. Even though these estimations were based on a limited number of experiments, the higher open probability found for KcsA-R64A agrees with data obtained by the group of Perozo [19]. Nevertheless, more systematic experiments need to be performed before we can draw solid conclusions with regard to the percentage of HOP and LOP activity in the KcsA-R64 mutants.

Functional stability of KcsA-R64 mutants

It was found that 85 percent of the R64D mutant channels closes shortly after initiating measurements. This probably correlates with difficulties to undergo the transition between the closed and open conformation. For KcsA-WT is has been found with tryptophan fluorescence quenching experiments that at least three out of four lipid binding pockets need to be occupied by anionic lipids in order for the channel to open [18]. Based on this study we speculate that lipids bind transiently to the binding pockets in order to facilitate opening

(Fig. 6 middle panel left) and closing *(Fig. 6 top panel left)* of the channel. Deactivation in some patches of KcsA-WT is probably due to the absence of lipids in the binding pockets (*Fig. 6 lower panel left)*. As described previously *(chapter 4)* it is likely that KcsA-R64A and particularly KcsA-R64D have an altered lipid binding ability, and most likely less lipids will bind due to the formation of electrostatically favourable salt bridges between adjacent monomers. We propose that in KcsA-R64D, after opening, any bound lipids lose their interaction with the lipid binding site to enable closure of the channel. Thereby they enable D64 and R89 to form an intermolecular salt bridge, consequently enhancing tetramer stability. As a final consequence, “new” lipids will be prohibited to bind to the lipid binding pocket, thereby disabling channel opening *(Fig. 6 lower panel on the right).* The remaining percentage of channels that remains constitutively active, thereby behaving like KcsA-WT, may then keep a constant amount of at least three anionic lipids bound, which only allows for one electrostatic interaction to form *(Fig. 6 middle panel right).* These channels will not gain in tetramer stability as assayed by TFE titration and gel shift assays, and this might explain the error found in these experiments *(chapter 4).* Moreover it would imply that the selectivity filter in these channels is constitutively active, indicating that the observed gating must solely originate from the pH dependent gate located at the C-terminus [7, 9].

For KcsA-R64A we suggest a slightly different mechanism since no salt-bridge can be formed between A64 and R89. We propose that in this mutant lipids are able to bind to the binding site in a similar way compared to KcsA-WT *(Fig. 6 top panel in the middle),* with the only difference that in KcsA-R64A only residue R89, and not A64, supplies an electrostatic interaction site. The lack of charge on A64 in addition to the bigger space that A64 generates in the binding pocket compared to R64 might cause that besides the relatively small 'standard' lipids (e.g. PG, PE and PA) in KcsA-R64A also other lipids with larger head groups such as phosphatidylinositol (PI), which is present in large amounts in the asolectin used, are able to bind to these pockets. Since tetramer stability of all three proteins in asolectin is similar to that in virtually all other bilayers tested, these non-conventional lipids apparently do not influence tetramer stability. This is explained by a model in which the KcsA tetramer is stabilized by lipids at three different sites: (I) The positively charged protein surface, where R89 from one subunit and R64 and T61 from the adjacent subunit hydrogen bond to the polar headgroups of the phospholipids, (II) The gorge formed by L86 and P63, defining a narrow entrance for the acyl chains, and (III) A hydrophobic pocket formed by different residues from the TMII of a subunit and from the pore helix of the adjacent subunit [31]. Since in our KcsA-R64 mutants only the first interaction site is altered it is seems that this basic tetramer stability is governed by binding site (II) and (III), whereas the enhancements of this stability, as seen for PA in KcsA-WT, take place at binding site (I).

Even though these non-conventional lipids do not influence the general tetramer stability, their interaction with the channel most likely differs from that of the regular lipids bound. For example, a larger head group may inhibit certain structural rearrangements, thereby prohibiting the channels to be able to open *(Fig. 6 lower panel in the middle)*. For the remainder percentage of the KcsA-

R64A channels that behave completely like KcsA-WT the mechanism may be similar as proposed for KcsA-R64D *(Fig.6 middle panel in middle).*

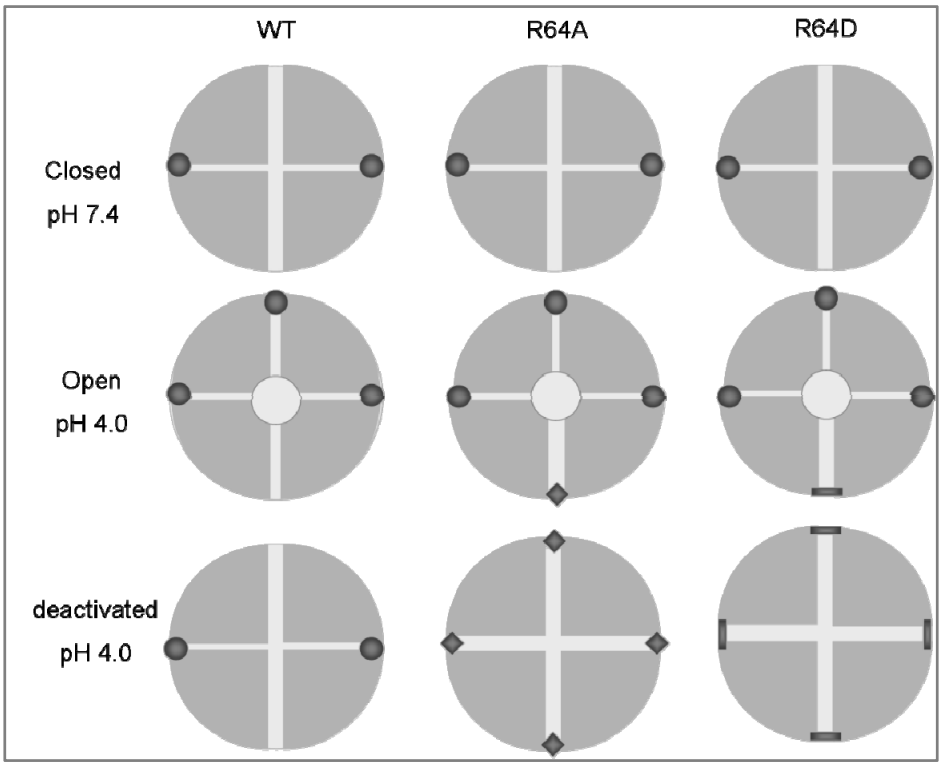


Figure 6: Gating model for KcsA. Cartoon representing a top view on the channels. KcsA-WT (left) allows binding of two lipids (●) at high pH. Upon lowering of the pH structural rearrangements take place, allowing binding of three or four lipids to the binding sites, thereby consequently resulting in opening of the extracellular gate (white circle). For KcsA-R64A (middle) and KcsA-R64D (right) we propose a similar gating mechanism as for KcsA-WT in which two lipids are bound and upon lowering of pH initially three or four lipids are able to bind, resulting in open channels at pH 4.0. However it has been found that a percentage of all channels deactivates after a short period of time. During deactivation in KcsA-WT, and partly also in both KcsA-R64 mutants, the channels are closed. Indicating that less than three binding pockets are occupied (lower panel left). For KcsA-R64A we additionally postulate that since the charge of the arginine is no longer present, and the alanine generates more space for a lipid head group it is likely that, in time, a wider variety of lipids is able to bind (♦). These lipids might have a different interaction with the binding pocket compared to the anionic lipids that are suggested to normally bind, thereby making the channels less stable which results in deactivation of a higher percentage of channels compared to KcsA-WT (lower panel middle). In KcsA-R64D a similar process as for KcsA-R64A is proposed, only in KcsA-R64D the instability of the channels is likely caused by the salt bridge formation (—) between D64 of one and R89 of the adjacent monomer. This, in time, will result in deactivation of most channels (lower panel right).

The mechanisms described above agrees with the observation that silencing of the channels by NaCl as well as by the Shaker B peptide is not altered, since in the remainder percentage of channels on which these experiments were conducted “original” lipids are thought to be present. However it would be interesting to further investigate the silencing of NaCl and the Shaker B peptide on these mutants channels, since subtle differences may become apparent when a statistically relevant amount of experiments is performed.

Future perspectives

The differences in functional properties between KcsA-WT and the KcsA-R64 mutants imply that R64 is involved gating. Sofar we only have a very speculative model *(Figure 6)* in which we propose that the structural differences between these proteins form the basis of their differential gating behavior as described above. To obtain more insight in the exact mechanism it would be interesting to test the relevance of the formation of a salt bridge by R64D and R89 of the adjacent monomer by performing similar experiments using KcsA with a mutated R89. Furthermore, insight into the lipid specificity of the gating process could be obtained by performing functional experiments in lipid bilayers of well-defined composition, as described for the TFE stability assays. Such experiments might also allow to further investigate tetramer stability and gating behavior as possibly coupled processes. However obtaining giant liposomes which are sufficiently stable for patch clamp studies are as yet very difficult to obtain. An alternative, simplified approach in this direction could be the use vesicles of asolectin in which the lipid composition is modified by systematic addition of well-defined lipids (Gonzalez-Ros unpublished data).

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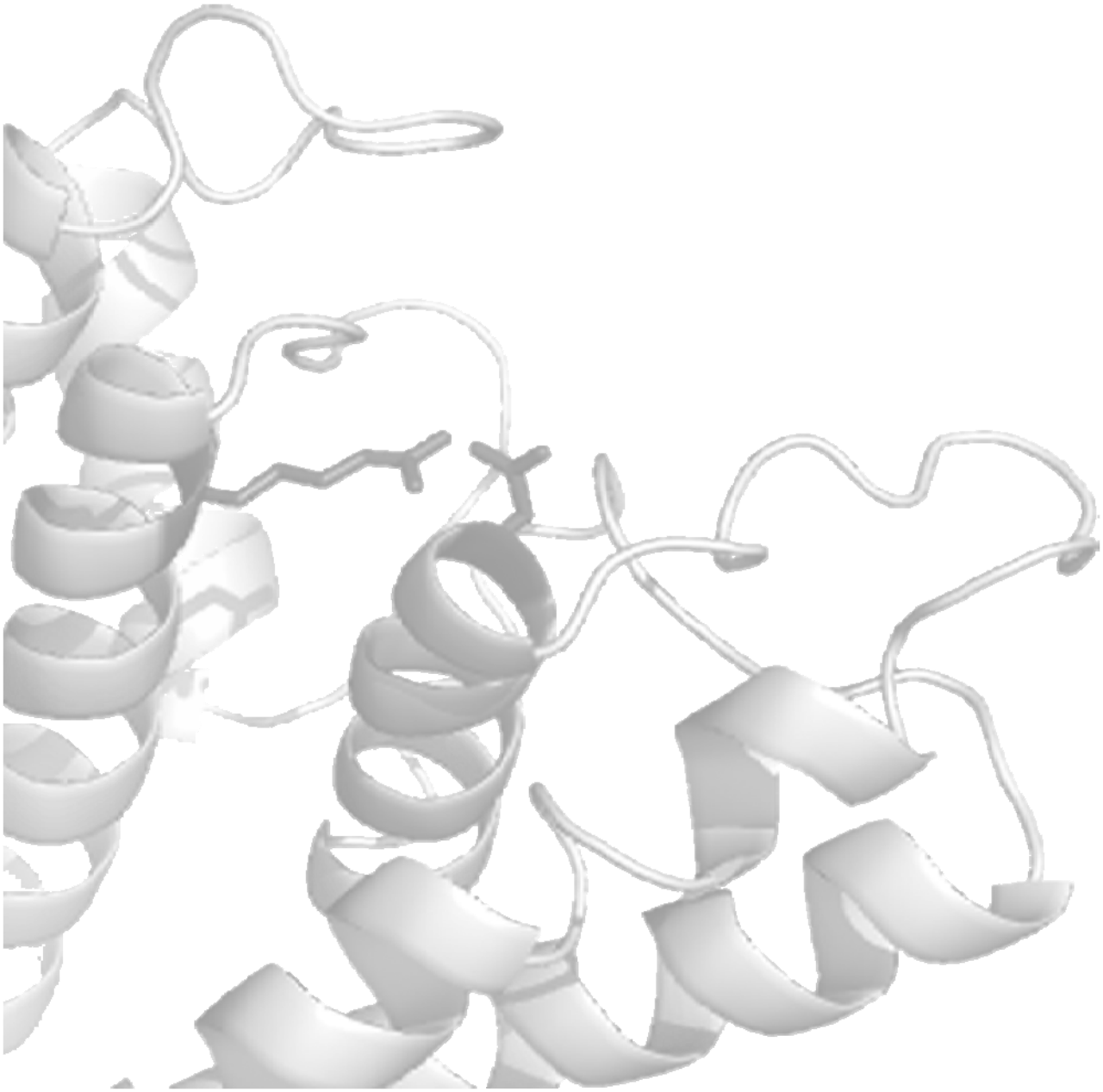
Chapter 6

General discussion



Many membrane proteins exist as oligomers. Such oligomers play an important role in numerous cellular processes such as ion transport, energy transduction and cell wall synthesis. However, many questions are still unanswered regarding oligomeric membrane protein assembly, stability and functioning. Specifically it remains unclear how lipids are involved in these processes.

The oligomeric potassium channel KcsA has proven to be an attractive model for addressing questions of how membrane lipids can affect assembly, function and stability of oligomeric membrane proteins. The reasons are the following. First, KcsA forms a stable homotetramer, even in SDS at 80°C [1], allowing straightforward analysis of the presence of intact tetramers by gel-electrophoresis. Second, the structure of the tetramer is known [2, 3], facilitating development of models to understand interactions between subunits and effects of lipids on a molecular level. Third, tetramer formation can be studied both *in vivo* and *in vitro* upon reconstitution in model membranes, allowing systematic variation of lipid composition [4]. Fourth, the importance of lipids for various process has been demonstrated, e.g. it has been found that lipids are involved in tetramer assembly, stability and even gating [4-7]. Finally, the functional properties of KcsA have been studied extensively, e.g. by preparation of specific KcsA mutants and by testing effects of voltage and pH [7-10].

The research in this thesis describes the way in which various properties of the KcsA tetramer can be influenced by intrinsic protein properties as well as by specific lipids. Different types of mutations within KcsA were made and in chapters 2, 3 and 4 their effects on tetramer stability were tested in a variety of different lipid systems. In chapter 2 it was found that replacing the inner or outer helix of KcsA by an alanine/leucine stretch or deleting significant parts of the selectivity filter does not prohibit the protein from forming tetramers. This suggested that the C-terminus is a key player in tetramer assembly, whereas the presence of the selectivity filter in combination with interactions between intramembrane segments of the protein, possibly mediated by lipids, are likely to add to the process of maintaining tetramer stability. In chapter 3 the role of lipids was further investigated and it was established that the anionic phospholipid PA has a specific, charge dependent interaction with KcsA, thereby stabilizing the tetramer. The results suggested that the specific charge properties of PA are the key to enhancing stabilization and it was proposed that the positively charged residues R64 and R89 which are thought to be involved in lipid binding [5, 11-13], represent the binding partners of PA in tetramer stabilization. This stabilizing effect by PA was not observed in the chimeric channel KcsA-Kv1.3, indicating that the altered loop in this chimera plays a role in the loss of interaction with PA. Since the basic residue R64 in KcsA is mutated into an aspartic acid in KcsA-Kv1.3 and since additionally R64 had been modeled to be part of a specific lipid binding site via electrostatic interactions [11], it was speculated that possibly this mutation alone could be responsible for the loss of stabilization by PA in KcsA-Kv1.3. To test this, we introduced the R64D mutation in KcsA-WT in chapter 4. Surprisingly, we found that this mutation leads to a significant increase in tetramer stability compared to KcsA-WT. This enhancement of stability is most likely due to a salt bridge that is formed between D64 and R89 of the adjacent monomer, thereby

mimicking the electrostatic interactions that otherwise most likely occur through lipid binding.

In chapter 5 we compared the functional properties of the different KcsA-R64 mutants and we found that KcsA-R64D has similar functional properties as KcsA-WT, but with one important difference. The KcsA-R64 mutants are functionally less stable compared to KcsA-WT and most channels close within a few minutes after activation without reopening. Together this implies that lipid binding is involved in structural stabilization of the tetramer as well as in allowing opening and closing of the channel, thereby making it likely that these processes are coupled. Based on these findings we propose in chapter 5 that lipids are part of the mechanism that enables KcsA to transform from the open to closed conformation and vice versa.

In the following section, we will discuss the findings above and their implications in more detail. First, the implications of the results described in chapter 2 on intrinsic protein properties that govern KcsA with its unique stability will be discussed with regard to a potential model to investigate the assembly and stability of oligomeric membrane proteins in a systematic way. Subsequently, the influence of lipids and protein properties on KcsA tetramer stability and gating, as studied in chapters 3, 4 and 5, will be discussed and a gating model for KcsA will be proposed in which lipids play a prominent role. The results will be discussed in relation to existing literature data.

KcsA mutants as model to investigate properties of oligomeric membrane proteins

In chapter 2 it was found that stable tetramers of KcsA can still be formed when both transmembrane segments in each monomer are replaced by a stretch of alternating leucines and alanines or even when the entire region connecting the two transmembrane helices is deleted, including the loop and selectivity filter. This indicates that the C-terminus, which according to X-ray data forms a long intracellular coiled-coil in the cytoplasm [3], must be a key feature in tetramer assembly of the potassium channel KcsA. An important implication of this observation is that it might allow us to create model proteins with any desired transmembrane sequence which still can assemble by itself into stable oligomeric structures in *E.coli* membranes. Thus, by establishing the minimal requirements for the formation of such a tetramer, we could develop a unique model system for highly systematic studies on factors involved in membrane protein oligomerization, oligomer stability, and protein-lipid interactions in general. This would be complementary to studies on the much simpler system of the so-called WALP peptides, which are a family of synthetic α-helical transmembrane peptides that are used as models for membrane spanning segments of single span membrane proteins to study protein-lipid and protein-protein interactions [14, 15].

A convenient starting point for such a system would be a model protein based on KcsA, in which the pore region is deleted in combination with two alanine/leucine stretches replacing the original helices. Simple variation of these helices would then allow systematic investigation of the possible involvement of intrinsic protein properties, such as hydrophobic length and hydrophobicity of the transmembrane segments, in assembly and stability. Another interesting

protein feature that could be assessed is the role of the flanking residues at the membrane/water interface which are suggested to function as anchors [16, 17]. The role of these residues in tetramer stability and function can be examined by mutating these residues, similar as has been done for the synthetic α-helical membrane spanning model peptides WALP and KALP [14, 18]. Also the location of the tryptophans could be altered to study if the position of these residues at the interface of the membrane is of importance for assembly as previously suggested for the prokaryotic inward rectifying potassium channel Kirbac1.1 [19].

Another interesting option would be to find out which properties are required to prepare a 'minimal channel' by removing (part of) the N-terminal helix or C-terminus. In KcsA-WT these are suggested to contain information necessary for assembly [20] and stability (e.g. chapter 2) and hence their systematic removal would allow further unraveling of key features for assembly and stability within KcsA. Alternatively one could even consider the viral channel KcV [21] as a starting point for designing a general 'minimal' model protein [22], since KcV (55 kD) is smaller compared to KcsA (72 kD). KcV lacks an N-terminal helix and has a much shorter C-terminal helix bundle [23], which would make it easier to transform it into a minimal channel. However there are also some disadvantages coupled to using this protein. Most importantly, no crystal structure is as yet available for this channel, making it more difficult to interpret results on a structural basis. Furthermore, the expression of this channel is more complex than that of KcsA, since, to our knowledge, it is still not possible to express this protein in *E.coli* [21, 24]. Finally it should be noted that for all functional potassium channels also aqueous conditions, such as the concentration of specific ions [8, 25-27] and pH [28, 29] should be taken into account.

In addition to changing protein properties to study assembly and stability of oligomeric proteins, one could also vary the membrane environment. This is particularly interesting since lipids are known to play a role in the assembly and stability of KcsA-WT [4, 6, 13, 30-32]. In our *in vivo* approach the membrane environment is formed by the organism in which the protein is expressed. This means that the use of bacteria or yeast strains altered in their lipid metabolism could be useful to obtain general information of the influence of certain lipids on assembly and stability. Such an approach would bypass the necessity of protein purification, which is a advantage when problems in protein expression are encountered. Examples of such strains are the *E.coli* mutant strains AD93 [33] and AH930 [34] which lack the ability to synthesize PE and the fatty acid auxotrophic strain K1059 [35].

Alternatively, if purification of the oligomeric protein from the host membrane is successful, the protein can be examined in a detergent environment e.g. to probe the presence of native, tightly bound lipids in the oligomeric complex. Alternatively, the purified proteins can be reconstituted into large unilamellar vesicles of choice. This can entail that purified membrane vesicles of certain organisms such as *E.coli* [36] are used, or that the LUVs are composed of synthetic lipids as described in this thesis. These synthetic lipids can be chosen in such a way that the influence of different head groups or hydrophobic tail length can be assessed. The latter can also be of interest in combination with altering the length of the helices as described above, to

investigate hydrophobic mismatch situations. If purification is a problem, then the *in vitro* transcription translation approach offers yet another useful alternative to vary the lipid environment. In this approach the protein is synthesized using a cell-free expression system in which acceptor vesicles are present that can be varied in the same way as described above for LUVs in the reconstitution process. This system offers the advantage that next to assembly into oligomers and oligomer stability also biosynthetic insertion into the acceptor vesicles can be monitored [4], which is prohibited in the reconstitution approach.

The influence of lipids on oligomeric membrane proteins

Lipids are known to affect the function of many different channels [37, 38]. In chapters 3, 4 and 5 of this thesis we established that in KcsA lipids are involved in tetramer stability and function. Here it will be discussed how these findings correlate to what has been found for other channels, and additionally, if this influence of lipids could be a general regulatory mechanism.

The mechanism by which lipids are involved in stability and gating of KcsA most likely can be extrapolated to other (potassium)channels with a necessity of lipids for their function, such as several Kir [39] [40] and Kv [41] channels. It seems likely that the mechanism of function, and thereby the lipid binding site, amongst these channels is more or less conserved in evolution. However, our findings that KcsA is stabilized by PA, whereas the chimeric channel KcsA-Kv1.3 with different amino acids at the proposed interaction site is not *(chapter 3),* emphasizes that the lipid binding mechanism can be altered. On the other hand, also in this chimeric channel the presence of especially PE and PA generates changes in the two gating regions [42]. This implies that although the lipid binding properties of different potassium channels might differ, the general role of these lipids in gating might be similar.

Besides potassium channels, lipids are also found to influence other channels such as the channel of large conductance MscL [43]. Recently it even has been established that this interaction is specific towards anionic lipids, whereas the interaction with zwitterionic lipids depends of the ability of the lipid to interact via hydrogen bonding. It was found that the interaction site for these lipids lies within the positively charged Arg-104, Lys-105, Lys-106 cluster located on the cytoplasmic site of the protein [44]. Although the location of the binding pocket in MscL is different compared to that of KcsA, the general features are the same. Namely, the binding pocket consists of several positively charged residues which preferably interact with anionic lipids or zwitterionic lipids with the ability to form hydrogen bonds. In addition in both cases lipid binding seems to be a trigger for (rapid) opening of the channel indicating a role as co-factor in gating. The observation that lipid binding is involved in a similar way in the gating of two very different types of oligomeric channels, strengthens our hypothesis that lipids might also act as co-factors in numerous other channels and oligomeric complexes of other function.

Biological implications of PA binding

As described above, features such as positively charged residues in the interaction site and anionic lipids as the most desired occupants seem to be general to lipid binding. However, also other factors might play a role. For

instance for KcsA it has been found that alkyl sulfates with a minimum chain length of 10 carbon atoms can stabilize tetramer stability [13] indicating that also the interaction of the hydrophobic tail with the protein is of importance.

Besides the features of the lipids and the protein involved, it is of importance to look at the biological context of the interactions. For instance, we found that PA has a stabilizing effect on the KcsA tetramer. However the presence of PA in the average bacterial membrane is very low, implying that *in vivo* the chance that a PA molecule actually comes in contact with an available lipid binding site is relatively low. On the other hand it has been suggested that PA has a signaling function, as described for the CTR1 protein kinase in plants [45]. Not much is known about PA as a signal molecule in bacteria, however it has been found to be involved in various signaling pathways in plants [46, 47] and mammals [48, 49]. In these pathways PA levels will increase upon stress thereby triggering several rescuing mechanisms. It seems attractive to imagine that an increase of PA levels, caused by a certain stress factor, can lead to an increase of PA binding to KcsA. The strong binding of PA to the channel subsequently could cause slower exchange of lipids in the binding pockets, thereby opening or closing the channels for a longer period of time.

In addition to the low levels of PA in bacterial membranes it also has to be noted that the native host of KcsA: *Streptomyces lividans*, has a different membrane composition than the *E.coli* bacterium in which KcsA is expressed under laboratory conditions The lipid composition of *Streptomyces lividans* has not been investigated yet. However, the lipid composition of some of its close relatives, *Streptomyces hygroscopicus* and *Streptomyces ambofaciens* was shown to contain much more anionic lipids compared to *E.coli* [50, 51]. This might indicate that the low opening probability found for KcsA in *E.coli* may in fact be an artifact of the lipid environment.

Model for KcsA gating

As discussed in chapter 4, we propose that besides influencing tetramer stability and gating, lipids bound to KcsA also cause an allosteric effect in which lipid binding to one monomer-monomer interface makes lipid binding to the adjacent monomer-monomer interface less favorable. This is supported by mass spectrometry experiments in which the KcsA tetramer dissociates into two dimers, rather than into monomers, or into a monomer and trimer. Such an allosteric effect was also suggested for the potassium channel KirBac3.1, based on atomic force microscopy experiments, which indicated that upon ligand binding the four cytoplasmic domains subdivide into two functional dimers, in which one of the monomers undergoes a larger displacement than the other [40]. Thus, the presence of an allosteric effect within the tetramer is not unique to KcsA, and we suggest that in both cases this effect may be caused by lipids. Additionally in chapter 5 we propose a model in which lipids are involved in the regulation of gating. Here we will merge these two models into a full lipid dependent gating mechanism for KcsA *(Fig. 1)*.

In this gating model the basis is formed by the suggested allosteric binding mechanism for stability in which typically two lipids are bound in opposite binding pockets. This would leave the two other binding sites empty and hence it would keep the channel closed (*Fig1 left*), since at least three of the binding sites need to be occupied by lipids in order for the channel to open

[7]. It is likely that the ability of the two empty binding sites to bind lipids is tightly regulated. It can be speculated that by the binding of lipids to two opposite binding sites, the conformation of the empty binding sites is such that lipid binding is less favorable. However upon an external stimulus, such as lowering of pH, conformational rearrangements e.g. in the hinge region of the protein may occur (*Fig. 1 middle*). Here we suggest a model in which these rearrangements (in)directly influence the conformation at the lipid binding sites, thereby allowing lipids to bind to the two empty pockets. Upon lipid binding to at least three binding sites rearrangements in the selectivity filter might take place in order to completely open the channel (*Fig. 1 right*). This model seems to contradict the findings by Perozo et al. that suggest that the coupling between the inner helical bundle and the selectivity filter relies on straightforward mechanical deformation [52]. However in their study the (subtle) rearrangements in the lipid binding pocket, as suggested in our model, were probably restricted by the binding of Fab fragments in close proximity of the pocket, and therefore not incorporated in their model.

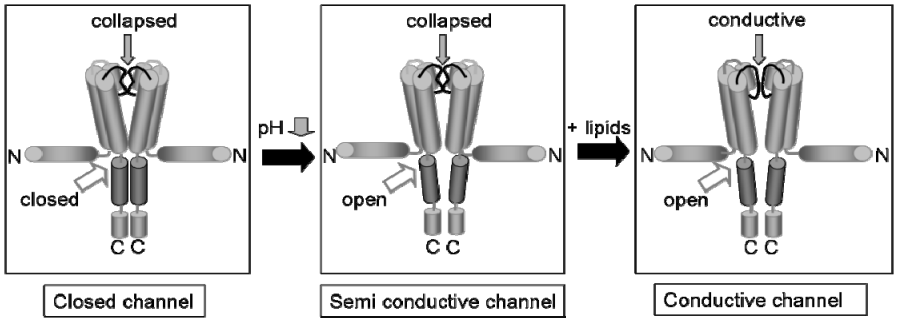


Figure 1: Proposed model for sequential opening of both gates in KcsA. The selectivity filter is indicated with a grey arrow, and the gate at the inner membrane interface with a white arrow. In general most channels are closed (left). Upon a decrease in pH below pH 5.0 the hinge region (dark helix) undergoes structural rearrangements, thereby opening the gate at the inner membrane interface (middle). By opening this gate structural rearrangements take place at the lipid binding sites, thereby allowing more than two lipids to bind. Upon this lipid binding the selectivity filter (Black line) will obtain its conductive form, consequently opening the channel (right). Two monomers are depicted for clarity.

Our model would imply that the pH is the main stimulus for channel opening and that lipid binding is the link between the opening of the hinge region gate and the gate at the selectivity filter by coupling of the two gates. When the hinge region closes, lipids are released and the selectivity filter collapses. It has to be mentioned that other factors, such as the presence of potassium in the selectivity filter, are also key to the final conductive state of the channel. Our proposed gating mechanism is supported by suggested mechanisms based on recent solid state NMR studies [53, 54]. In these studies structural rearrangements in the lipid binding pocket are observed in addition to rearrangements in the hinge region upon lowering pH, suggesting coordinated and sequential opening and closing of both gates.

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Dutch summary
(Nederlandse samenvatting)

De invloed van lipiden op een kaliumkanaal

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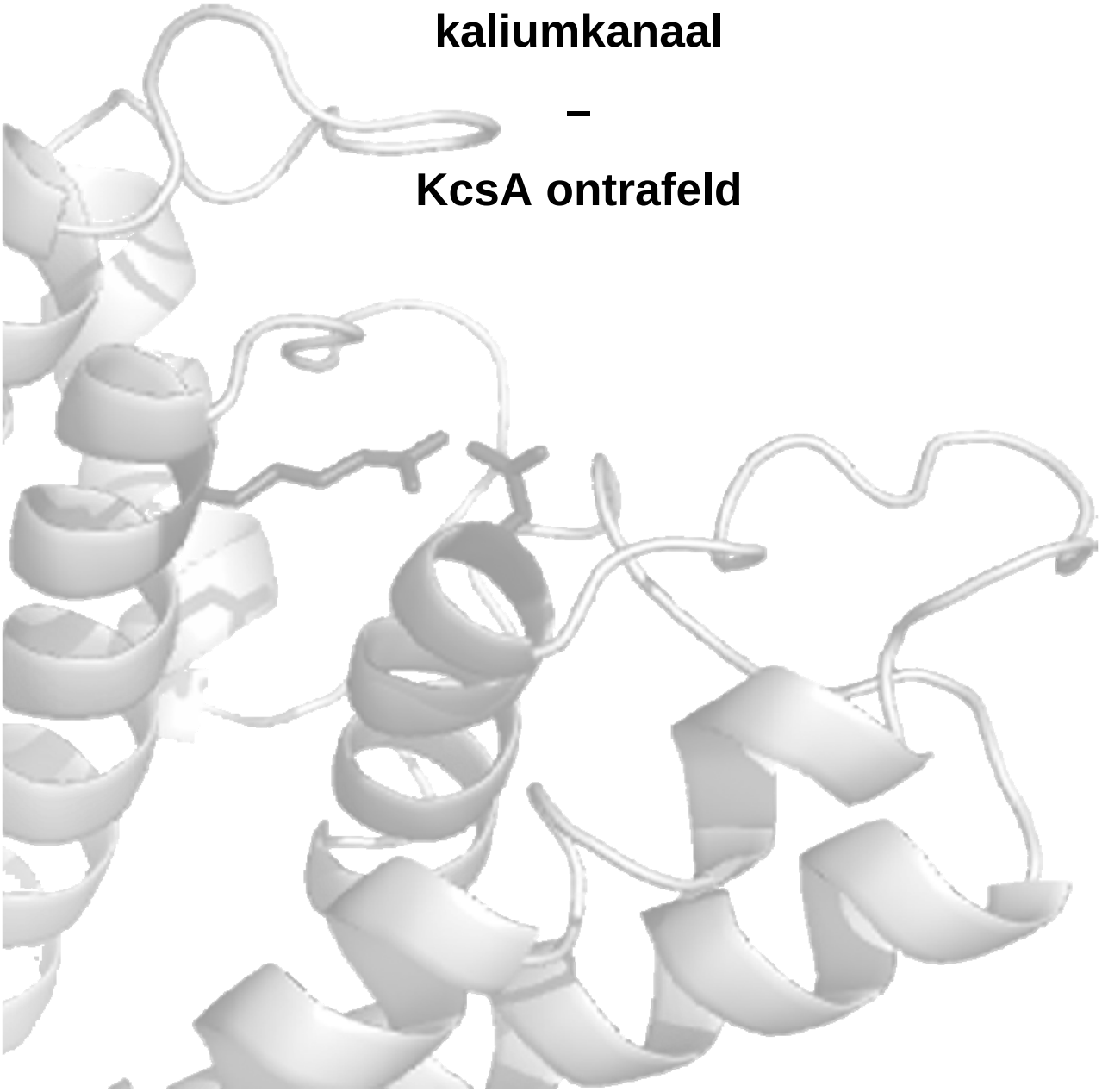
KcsA ontrafeld



Membranen

Alle organismen bestaan uit een of meerdere cellen. Al deze cellen hebben ongeveer dezelfde algemene structuur met vergelijkbare componenten. Deze overeenkomsten zorgen ervoor dat we bijvoorbeeld bacteriën kunnen gebruiken om bepaalde biologische processen te bestuderen die in mensen op een soortgelijke manier verlopen.

Elke cel wordt omgeven door een beschermend omhulsel: de celmembraan, dat de inhoud van de cel scheidt van de omgeving. Naast de celmembraan kunnen cellen ook interne membranen bevatten die compartimenten in de cel met een specifieke functie omsluiten. Een membraan is opgebouwd uit vele individuele moleculen, die in twee klassen kunnen worden ingedeeld: lipiden en eiwitten.

Een lipide is een molecuul met een hydrofiele (wateroplosbare) kop en een hydrofobe (niet wateroplosbare) staart. In de membraan vormen lipiden een dubbellaag waarbij de hydrofobe staarten naar elkaar toe zijn gericht en de hydrofiele koppen in contact staan met de waterige omgeving. Een grote variatie in eigenschappen van zowel de koppen als de staarten zorgt voor een grote verscheidenheid aan lipiden in de membraan. Deze lipiden vormen de werkelijke barrière tussen de inhoud van de cel en de omgeving. Deze barrière heeft als voornaamste functie het beschermen van de cel. Het is echter ook noodzakelijk om moleculen en informatie uit te wisselen met deze omgeving. Zo moeten voedingsstoffen naar binnen kunnen en afvalstoffen naar buiten. Ook moet een cel zich kunnen aanpassen aan veranderingen in de omgeving door signalen op te vangen. Deze functies worden uitgevoerd door eiwitten.

Een eiwit is opgebouwd uit een lange ketting van bouwstenen (aminozuren). Deze ketting neemt een ingewikkelde, opgevouwen structuur aan, die vooral bepaald wordt door de eigenschappen van de aminozuren. De manier waarop het eiwit is gevouwen bepaalt ook in grote mate de functionaliteit van het eiwit. Eiwitten kunnen op verschillende manieren met de membraan verbonden zijn. Sommigen eiwitten steken dwars door de membraan heen en worden integrale membraaneiwitten genoemd. Daarnaast zijn er ook eiwitten die aan de membraan hangen zonder er doorheen te gaan. Deze eiwitten worden perifere membraaneiwitten genoemd.

Veel membraaneiwitten werken niet als enkel eiwit maar in een complex van eiwitten. Zo'n complex kan bestaan uit een veelvoud van eenzelfde eiwit, maar ook uit een aantal compleet verschillende eiwitten. Samen vervullen deze eiwitten dan hun specifieke functie. In dit proefschrift staat een integraal membraaneiwitcomplex centraal dat uit vier dezelfde eiwitten is opgebouwd (homotetrameer) en dat functioneert als een kanaal dat selectief kalium moleculen doorlaat. Dit specifieke complex wordt KcsA genoemd.

KcsA komt uit de in modder levende bacterie *Streptomyces lividans*. KcsA is een relatief klein en simpel kaliumkanaal met karakteristieken die ook in veel humane kaliumkanalen aanwezig zijn. Deze eigenschappen maken KcsA een geschikt modeleiwit om een beter inzicht te krijgen in het algemene functioneren van kaliumkanalen. Onderzoek naar het functioneren van kaliumkanalen is van belang omdat dit kanaal, en vooral het niet (goed) functioneren daarvan, aan de basis ligt van verschillenden aandoeningen zoals hartfalen en diabetes.

Onderzoeksvragen

KcsA is een kaliumkanaal waaraan al veel onderzoek is gedaan. Zo heeft Roderick MacKinnon in 2003 een Nobelprijs gekregen voor het oplossen van de kristalstructuur (driedimensionale vouwing) van KcsA. Hoewel deze kristalstructuur het onderzoek naar kaliumkanalen een stuk makkelijker en aantrekkelijker heeft gemaakt, worden veel aspecten van deze kanalen nog steeds niet goed begrepen.

Omdat KcsA een integraal membraaneiwitcomplex is en dus omgeven is door lipiden, is het aannemelijk dat deze lipiden het kaliumkanaal kunnen beïnvloeden. Bovendien zijn in de kristalstructuur van KcsA lipiden gevonden, wat betekent dat deze sterk aan het eiwit gebonden zitten. Verder is het bekend dat anionische (negatief geladen) lipiden nodig zijn voor het functioneren van het kanaal. Het is echter niet bekend hoe deze lipiden KcsA precies beïnvloeden.

In dit proefschrift is daarom een begin gemaakt met het ontwikkelen van een modelsysteem, gebaseerd op KcsA, waarmee verschillende karakteristieken (assemblage, stabiliteit en lipide-afhankelijkheid) van eiwitcomplexen (oligomeren) systematisch kunnen worden onderzocht. Verder is onderzoek gedaan naar specifieke eigenschappen van KcsA die wellicht vertaald kunnen worden naar andere kaliumkanalen. Zo is er bepaald of (specifieke) lipiden invloed hebben op de stabiliteit van de KcsA tetrameer. Daarnaast is onderzocht of deze invloed wordt uitgeoefend door middel van een specifieke interactie met de KcsA tetrameer of dat deze via een meer algemeen mechanisme tot stand komt. Bovendien is er ook gekeken of de binding van lipiden van invloed is op de functionaliteit van het kanaal.

Methode

Om het algemene model voor het bestuderen van oligomere eiwitten te realiseren is er gekeken welke delen van KcsA essentieel zijn voor het vormen van een stabiele tetrameer. Dit is gedaan door een PCR (polymerase chain reaction) methode op te zetten waarmee de transmembraanhelices (de stukken die door de membraan steken) van KcsA vervangen zijn door een repeterende alanine leucine aminozuurketen. Naast het vervangen van deze helices zijn ook grote delen aan de binnenkant van het kanaal verwijderd om te kijken of deze delen van belang zijn voor tetrameer-formatie.

Om te kijken welke lipiden invloed hebben op de stabiliteit van de KcsA tetrameer is KcsA in de bacterie *E.coli* geproduceerd, om vervolgens het eiwit te zuiveren en in kunstmatige cellen te plaatsen. De lipidensamenstelling van deze kunstmatige cellen kan systematisch gekozen worden om zo de invloed van een bepaald lipide te onderzoeken. Vervolgens is de stabiliteit van de tetrameer bepaald door middel van een klein alcohol dat wordt toegevoegd aan deze kustmatige cellen met daarin KcsA. Bij een bepaalde hoeveelheid van dit alcohol zal dan het tetramere kanaal uiteenvallen in vier monomere eiwitten. Met deze methode kan het verschil in tetrameerstabiliteit in aanwezigheid van verschillende lipiden bepaald worden.

Vanuit de literatuur zijn er verschillende aanwijzingen dat lipiden een specifieke interactieplaats met de KcsA tetrameer aangaan. Door middel van een KcsA mutant, waarin een aminozuur in deze verwachte interactieplaats is vervangen door verschillende andere aminozuren, is bepaald of dit aminozuur

inderdaad betrokken is bij de binding van lipiden. Vervolgens is er ook naar de invloed van lipiden gekeken op de stabiliteit van deze gemuteerde eiwitten.

De invloed van lipiden op de functionaliteit van KcsA is bestudeerd met de patch clamp techniek. Met deze techniek is door middel van de stroom die door het kanaal loopt bekeken of de gemuteerde eiwitten dezelfde hoeveelheid kalium doorlaten als het originele eiwit. Daarnaast is er ook gekeken of de gemuteerde kanalen nog steeds selectief zijn voor kalium, en dus geen andere moleculen doorlaten.

Hieronder volgt een korte samenvatting van dit onderzoek, zoals beschreven in hoofdstuk twee tot en met vijf van dit proefschrift.

De stabiliteit van de KcsA tetrameer is gebaseerd op meerdere eiwitkarakteristieken

Als eerste werd onderzocht welke delen van KcsA verantwoordelijk zijn voor het stabiliseren van de tetrameer. KcsA is opgebouwd uit een helix die tegen de binnenkant van de celmembraan ligt die overgaat in een helix die door de membraan steekt en uitmondt in het selectiviteitsfilter wat er voor zorgt dat alleen kalium door het kanaal past. Daarna volgt een tweede helix die door de membraan steekt en eindigt de aminozuursequentie met een groot gedeelte wat aan de binnenkant van de cel hangt (C-terminale deel). Uit eerder onderzoek is gebleken dat de aanwezigheid van dit C-terminale deel van KcsA essentieel is voor het vormen van stabiele tetrameren. Bovendien is aangetoond dat het binden van kalium aan het selectiviteitsfilter de stabiliteit van de tetrameer verhoogd.

In dit onderzoek is gekeken of de aminozuursequentie van beide transmembraanhelices van belang is voor de tetrameerstabiliteit. De eerste transmembraanhelix zit aan de buitenkant van het kanaal en heeft dus veel interactie met de lipiden in de membraan. Het is daarom denkbaar dat veranderingen in de aminozuren van deze helix leiden tot een ander interactiepatroon met de lipiden waardoor de stabiliteit van de tetrameer veranderd. Deze hypothese is getest door met een nieuwe PCR-methode de hele buitenste transmembraanhelix te vervangen door leucine en alanine-residuen. Deze residuen zijn gekozen omdat ze goede helixvormers zijn en daarmee dus de structuur van de transmembraanhelix intact laten. Bovendien hebben deze residuen geen grote zijketens of lading, wat betekent dat alle specifieke eigenschappen van de originele KcsA-aminozuren op deze manier worden verwijderd. Een soortgelijk experiment is uitgevoerd met de tweede transmembraanhelix van KcsA. Deze helix bevindt zich aan de binnenzijde van het kanaal. Dit betekent dat de aminozuren in deze helix vooral interacties aangaan met aminozuren van andere delen van het eiwit om zo de tetrameer te stabiliseren. Dit zou betekenen dat als de specifieke karakteristieken van deze aminozuren worden verwijderd, deze interacties niet meer kunnen plaatsvinden waardoor de stabiliteit van de tetrameer word aangetast.

Na het vervangen van een van de helices werd de darmbacterie *E.coli* gebruikt om het gemuteerde eiwit te produceren waarna op een eiwitgel de conformatie (monomeer/tetrameer) van het eiwit werd gecontroleerd. Allereerst werd al snel duidelijk dat de bacterie grote moeite had met het maken van de gemuteerde eiwitten. De hoeveelheid eiwit die geproduceerd werd lag veel lager dan de hoeveelheden die gebruikelijk zijn voor het originele eiwit. Verder

bleek ook dat de conformatie van het eiwit nogal varieerde per experiment. Tegen alle verwachtingen in werd er soms, voor beide mutanten, wel degelijk een stabiele tetrameer gevormd, maar ook de monomeren en andere conformaties waren vaak aanwezig. Om deze problemen met reproduceerbaarheid op te lossen is er veel tijd gestoken in het optimaliseren van de productie van het mutante eiwit, helaas zonder resultaat. Het belangrijkste wat we uit deze experimenten kunnen concluderen is dat de aminozuursequentie van beide helices niet essentieel is voor het vormen van KcsA tetrameren.

Naast het bestuderen van de invloed van de aminozuren van de transmembraanhelices is er ook gekeken of het selectiviteitsfilter van KcsA essentieel is voor het vormen van een tetrameer. Om dit te onderzoeken zijn er mutanten van KcsA gemaakt waarbij dan wel het selectiviteitsfilter alleen, dan wel het selectiviteitsfilter samen met aangrenzende aminozuurketens is verwijderd. Vervolgens is wederom gekeken welke conformatie van het eiwit werd gevormd na productie in de bacterie *E.coli.* Omdat al bekend was dat binding van kalium aan het selectiviteitsfilter de KcsA tetrameer stabiliseert, werd verwacht dat zonder selectiviteitsfilter de tetrameer wellicht niet meer gevormd zou kunnen worden. Het tegendeel bleek waar. Ook zonder de aanwezigheid van het selectiviteitsfilter is KcsA in sommige experimenten als tetrameer aanwezig. Echter, ook in deze experimenten bleken de verkregen eiwitconformaties niet reproduceerbaar.

Het lijkt er dus op dat zowel de aminozuursamenstelling van de helices als de aanwezigheid van het selectiviteitsfilter niet essentieel zijn voor de formatie van KcsA tetrameren. Daar moet echter wel aan toegevoegd worden dat er door de slechte reproduceerbaarheid van de experimenten er niets kan worden gezegd over de stabiliteit van deze mutante eiwitten ten opzichte van het originele eiwit. Daarnaast is het aannemelijk dat door de gemaakte mutaties, de tetramere eiwitten wellicht een andere conformatie hebben in vergelijking met het originele eiwit. Bovendien is het niet waarschijnlijk dat de mutante eiwitten, en dan vooral die zonder selectiviteitsfilter, nog een functioneel kanaal kunnen vormen.

Kortom, de C-terminus van KcsA blijkt de hoofdfactor te zijn bij het vormen van tetramere kanalen. Dit zou betekenen dat een eiwit bestaande uit de KcsA C-terminus met daaraan vast een transmembraangedeelte waarin beide helices zijn vervangen door alanines en leucines maar zonder selectiviteitsfilter, een soort minimale tetrameer zou vormen. Dit eiwit zou vervolgens als modeleiwit gebruikt kunnen worden om de basisprincipes van assemblage, stabiliteit en lipide-afhankelijkheid van oligomere eiwitten te onderzoeken

De moleculaire basis van KcsA tetrameer stabilisatie door PA

Als tweede is onderzocht welke lipiden een stabiliserend effect hebben op de KcsA tetrameer. Er was recentelijk al gevonden dat het lipide PA de KcsA tetrameer kon stabiliseren. In dit onderzoek wordt verder ingegaan op de aard van deze stabilisatie, en dan vooral op de kenmerken van de betrokken lipiden.

Om te beginnen is er gekeken of het stabiliserende effect op de KcsA tetrameer van PA specifiek is. Om dit te onderzoeken is KcsA in kunstmatige cellen geplaatst met een systematisch gekozen lipidensamenstelling. De

gekozen lipiden zijn lipiden die aanwezig zijn in de membraan van bacteriën. Als negatieve controle is het lipide PC gebruikt dat niet in bacteriën voorkomt. Stabiliteitsproeven op KcsA in deze kunstmatige cellen wijzen uit dat het stabiliserende effect van PA specifiek blijkt te zijn, omdat een soortgelijke stabilisatie niet plaatsvindt in de aanwezigheid van andere lipiden. Dit betekend dat PA specifieke eigenschappen moet hebben die in de andere lipiden ontbreken.

PA is een negatief geladen lipide met een zeer kleine kop. Door deze kleine kop gaan de staarten uit elkaar staan, waardoor het lipide een soort piramidevorm krijgt. De combinatie van de negatieve lading en de piramidevorm in hetzelfde lipide blijkt van essentieel belang te zijn voor het stabiliserende effect op KcsA. Dit wordt bevestigd doordat in cellen met lipiden die alleen een negatieve lading of een piramide vorm bevatten, of in zelfs cellen waarin een combinatie van deze twee eigenschappen in verschillende lipiden aanwezig is, dit stabiliserende effect niet gegenereerd wordt.

Om meer inzicht te krijgen in de manier waarop PA de KcsA tetrameer stabiliseert, zijn soortgelijke stabiliteitsproeven, in cellen met verschillende lipiden, uitgevoerd bij een lagere pH. Bij deze pH verandert de lading van PA naar neutraal terwijl de lading van alle andere lipiden hetzelfde blijft. Het blijkt dat bij deze pH het stabiliserende effect dat PA op de KcsA tetrameer heeft, volledig is verdwenen. Dit bevestigt dat de lading van PA een belangrijke rol speelt bij het stabiliseren van de tetrameer.

Verder is er ook gekeken naar het effect van PA op de tetrameer van een op KcsA gebaseerd kaliumkanaal: KcsA-Kv1.3. Dit kaliumkanaal is in principe hetzelfde als KcsA, alleen heeft het elf andere aminozuren in het stuk tussen de eerste transmembraanhelix en het selectiviteitsfilter. Het bleek dat de tetrameer van dit kanaal in alle lipiden dezelfde stabiliteit had. Dit betekent dat het stabiliserende effect van PA in dit kanaal niet aanwezig is. Het is daarom aannemelijk dat PA in het originele eiwit bindt aan een van de aminozuren die in KcsA-Kv1.3 veranderd is.

De rol van residu R64 in tetrameerstabiliteit en lipidebinding

Voorgaande bevindingen suggereren dat PA waarschijnlijk een specifieke interactieplaats heeft op het originele KcsA eiwit, die ligt in het stuk tussen de eerste helix en het selectiviteitsfilter. Op basis van deze bevindingen en een modelleringstudie die aminozuur R64 in deze regio aanwijst als mogelijke lipide bindingspartner zijn er KcsA mutanten gemaakt waarbij R64 is vervangen. Het aminozuur R (arginine) heeft een positieve lading. In onze eerst mutanten is dit residu vervangen door een neutraal aminozuur A (alanine) of door een negatief aminozuur D (aspartaatzuur).

Vervolgens werd de stabiliteit van deze mutanten getest in de bacterie zelf maar ook in kunstmatige cellen bestaande uit verschillende, systematisch gekozen, lipiden. Het bleek dat de KcsA-R64A mutant vrijwel dezelfde stabiliteit had in vergelijking met het originele eiwit. De KcsA-R64D mutant had echter een veel stabielere tetrameer in de aanwezigheid van vrijwel alle lipiden. Dit houdt in dat aminozuur R64 inderdaad betrokken is bij de stabilisatie van de tetrameer.

Volgens een modelleringstudie is naast aminozuur R64 ook aminozuur R89 betrokken bij de binding van lipiden. Dit betekend dat deze twee

aminozuren met hun positieve ladingen samen de kopgroep van een lipide zouden binden. Hoewel in de KcsA-R64A mutant een van die ladingen is verdwenen, word tetrameerstabiliteit, en dus waarschijnlijk ook de lipidebinding, hierdoor niet erg veranderd. In de KcsA-R64D mutant is het echter waarschijnlijk dat in plaats van het binden van een lipide, de negatieve lading van D64 en de positieve lading van R89 ervoor zorgen dat deze aminozuren aan elkaar binden (zoutbrug). Deze zoutbrug blijkt, op een soortgelijke manier als de binding van PA, de KcsA tetrameer te stabiliseren. Kortom deze lipidebindingsplaats blijkt cruciaal in het reguleren van de tetrameerstabiliteit.

Functionele eigenschappen van KcsA-R64 mutanten

Omdat aminozuur R64 van belang blijkt te zijn bij het reguleren van lipidebinding en daarmee tetrameerstabiliteit, is het interessant om te kijken of kanalen waarin R64 vervangen is nog wel op dezelfde manier functioneren in vergelijking met het originele kanaal. Om dit te testen zijn de twee KcsA-R64 mutanten in kunstmatige cellen geplaatst die opgebouwd zijn uit sojaboonlipiden. Er is gekozen voor sojaboonlipiden omdat dit vooralsnog het enige lipidesysteem is waarin deze experimenten kunnen worden uitgevoerd.

In deze experimenten werd vooral gekeken naar de functionele eigenschappen van deze gemuteerde eiwitten. In het algemeen is het gedrag van deze mutanten vrijwel hetzelfde als dat van het originele eiwit. Zo kunnen de kanalen zowel in een HOP (veel open kanalen) en een LOP (weinig open kanalen) patroon openen waarbij ook clustervorming van de kanalen kan optreden. Verder lijken alle kanalen kaliumspecifiek, en kunnen ze alle drie geblokkeerd worden door een bepaald peptide. Dit alles suggereert dat R64 niet betrokken is bij de basisfunctionaliteit van KcsA.

Echter de experimenten gaven ook een groot verschil aan tussen het functioneren van het originele eiwit en de beide mutanten. In het originele eiwit sluit er in de loop van de tijd een percentage kanalen irreversibel. In de KcsA-R64A mutant ligt dit percentage een beetje hoger, en voor de KcsA-R64D mutant zelfs vele malen hoger dan voor het originele eiwit. Dit geeft aan dat deze eiwitten waarschijnlijk minder stabiel zijn waardoor de kanalen makkelijker sluiten en/of moeilijker openen. Deze vinding sluit goed aan bij het idee dat lipiden betrokken zijn bij het stabiliseren van de tetrameer. Dit zou impliceren dat binding van lipiden aan de bindingsplaats de tetrameerstabiliteit een beetje verlaagt waardoor het kanaal kan openen en kalium door kan laten. En daarmee lijken lipiden dus een belangrijke rol te vervullen in de regulatie van het functioneren van KcsA.

Conclusie

In dit proefschrift is beschreven dat de KcsA tetrameer een zeer stabiel oligomeer eiwit is. Zelfs na ingrijpende mutaties in het transmembraan en kerngedeelte is het eiwit nog steeds in staat om een tetramere conformatie te verkrijgen. Dit duidt erop dat vooral het C-terminale deel van het eiwit van belang is voor tetrameerformatie. Door de verschillende mutaties in het transmembraandeel te combineren zou er een minimaal tetrameer eiwit gecreëerd worden dat als model zou kunnen dienen om oligomere eiwitten systematisch te bestuderen.

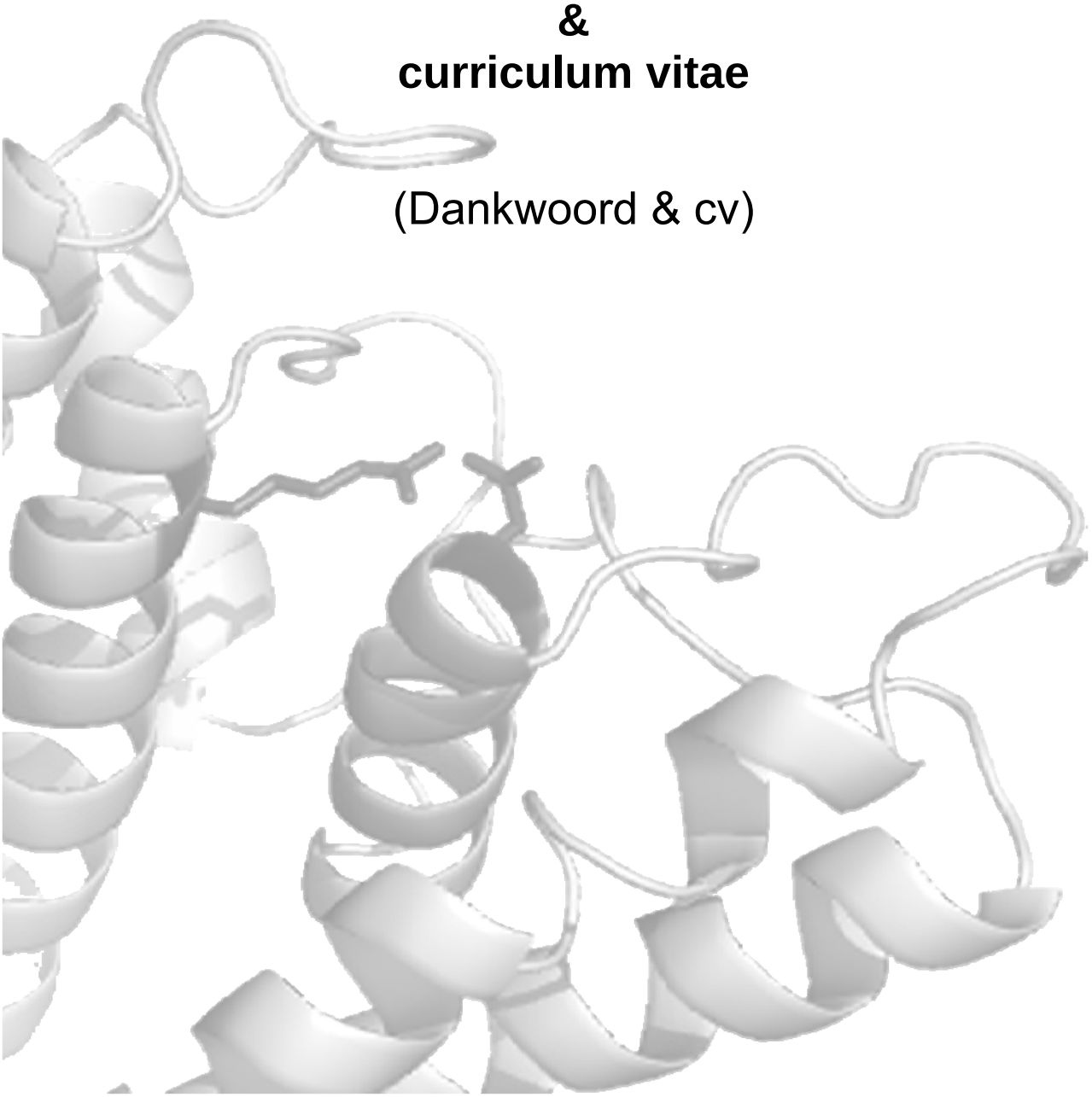
Verder is aangetoond dat lipiden, zoals verwacht, een grote invloed hebben op het kaliumkanaal KcsA. Zo is er aangetoond dat het lipide PA in staat is de stabiliteit van de KcsA tetrameer te vergroten en dat deze stabilisatie gebaseerd is op de negatieve lading en de potentie tot het vormen van waterstofbruggen van dit lipide. Daarnaast is vastgesteld dat aminozuur R64 betrokken is bij de specifieke binding van lipiden aangezien de tetrameerstabiliteit van KcsA drastisch verandert als dit aminozuur word vervangen door een ander aminozuur. Bovendien blijkt dat de eiwitten waarin R64 is vervangen, minder stabiel zijn in het uitoefenen van hun functie. Dit betekent dat de binding van lipiden waarschijnlijk ook van belang is voor het openen/sluiten van het kanaal en dus een regulerende functie heeft.

Ook voor andere kaliumkanalen, evenals voor andere kanalen, is bekend dat (anionische) lipiden van belang zijn voor functie. Dit maakt het aannemelijk dat het regulerende mechanisme van lipiden op de functie van KcsA, of een variatie hierop, ook aanwezig is in deze kanalen.

Acknowledgements

&
curriculum vitae

(Dankwoord & cv)



En toen was het klaar… Na ruim vier jaar op het lab en nog eens een half jaar achter de computer is mijn proefschrift eindelijk af. Ik ben er trots op en zie het als afsluiting van een intensieve maar toch wel mooie tijd. Ik ben dan ook veel mensen dankbaar voor hun steun en inzet tijdens mijn promotieonderzoek.

Allereerst Antoinette, ik ben je zeer dankbaar dat in de afgelopen jaren je deur altijd voor me open stond. Er was altijd wel even tijd om de laatste resultaten, een presentatie of andere (lab)issues te bespreken. Verder vond ik het prettig om met je te discussiëren over mijn experimenten en de rode lijn van elk hoofdstuk. Ik ben er trots op hoe we in de afgelopen zes maanden alle data een mooi plekje hebben kunnen geven in vier experimentele hoofdstukken.

Daarnaast wil ik Ben graag bedanken voor alle besprekingen gedurende mijn promotie. Ik heb er bewondering voor hoe jij meteen de implicaties van elk experiment in een groter geheel weet te plaatsen en terloops meteen de zwakke plekken benoemd. Ik hoop dat je emiritaat je de kans geeft om nog vaker af te reizen naar mooie (vogel) gebieden.

Ook Toon en Eefjan wil ik bedanken voor de ondersteuning en gezelligheid tijdens mijn promotie.

Next I want to thank Prof. Dr. Jose Manuel Gozalez-Ros for giving me the opportunity to visit his lab in Elche for two weeks. It was truly amazing to work in a lab were everyone works on the same protein with such enthusiasm and dedication. I especially want to thank Estefania, Asia and Maria Luisa for their help with performing and interpreting the patch clamp experiments. I hope the experiments will lead to a nice publication in the future.

In the category collaborations I would also like to thank Mark Baldus and Christian Ader from the NMR department for enabling me to work with the KcsA-Kv1.3 protein which caused a breakthrough in my research. I hope someday our findings will result in an nice story. In addition I would like to thank Deepak for his help with the pymol figures.

Ook wil ik Kees Versluis en Albert Heck van de afdeling massa spectrometrie bedanken voor hun bijdrage aan mijn proefschrift. Kees, echt super dat je in je laatste jaar voor je pensioen nog een nieuwe methode wilde opzetten om mijn eiwit te kunnen bestuderen. Er zijn door tijdsdruk helaas maar twee experimenten in mijn proefschrift terechtgekomen, maar ik ben er van overtuigd dat er nog vele mooie experimenten zullen volgen.

Verder wil ik natuurlijk alle medewerkers van de vakgroep biochemie van membranen bedanken voor de leerzame, kleurrijke maar vooral ook gezellige tijd op de zesde en later achtste verdieping. De afgelopen viereneenhalf jaar hebben jullie allemaal op je eigen manier mijn werkdagen ingekleurd.

Tania, ik vond het fijn dat wij min of meer tegelijk aan onze promotie zijn begonnen. Al snel waren we meer vrienden dan collega's en deelden we naast de aio kamer, het lief en leed van het promovenda zijn ook onze passie voor motorrijden. Bedankt voor je vriendschap en ik voel me vereerd dat je me straks als paranimf wil bijstaan.

Dan mijn andere Kamer genootjes. Andrea, bedankt voor alle gezelligheid. Onze skate-avondjes, het cola-bier en de Duitse versprekingen zal ik niet snel vergeten. Ik vond het een eer om jou paranimf te mogen zijn. Vincent, bedankt voor alle liederen, gedichten, tv-updates en Zegveld verhalen. Jij gaf BvM kleur en een stem. Jacob, al je bijnamen doe je eer aan. Bedankt voor een onwijs leuke tijd in Z602. Nick, altijd onverstoorbaar en goed met studenten (vooral uit India). Bedankt voor het mede organiseren van een super labdag. Jaques, it was pleasant and convenient to share a office with you since you know something about everything and a everything about some things. Thanks for all your help and support. Joost, Paulien en Remko, de nieuwste aanwinsten in de aio kamer. Bedankt voor jullie gezelligheid tijdens mijn laatste loodjes. Veel succes met jullie eigen promotietraject.

Ook de rest van het lab wil ik bedanken. Ten eerste Yvonne, ik vond het super om twee weken met jou op Corisca door te brengen. Verder wil je bedanken voor je luisterend oor en je sterke schouder. Ik ben er zeker van dat we in de komende jaren nog een keer gaan shoppen in Boston. Lucie I would like to thank for being a colleague, friend and roommate. I would never have been introduced to the kitkat-break, French vineyards and watching E.R. dvd's every evening if it wasn't for you, thanx. Irene D, bedankt voor alle praktische, organisatorische en emotionele ondersteuning. BvM zal na jou pensioen nooit meer hetzelfde zijn. Dick bedankt dat je altijd voor me klaar stond als er weer wat stuk was, of gewoon om even de dag door te nemen.

Martijn bedankt voor het introduceren van de vrijdagmiddag borrel en de bijbehorende doppinda's en gesprekken. Het was altijd fijn om de week even cynisch te analyseren onder het genot van een biertje. Heel veel succes met de laatste KcsA loodjes en ik hoop dat er mooie resultaten uitkomen. Mandy, bedankt voor alle gezelligheid en hulp op het lab. Zonder jou zou het vmt lab een stuk discipline en gezelligheid missen. Diana, wat fijn dat jij altijd wist waar ik alles kon vinden. Bedankt voor de gezelligheid en structuur op onze benches. Succes met de laatste loodjes! Het komt allemaal goed. Marlies, bedankt voor alle thee pauzes en de daarbij behorende gesprekken (vaak met HPLC-pauzes tussendoor). Heel veel succes met je nieuwe baan.

Dan de postdocs van het lab. Tami heel erg bedankt voor je hulp bij allerlei problemen. Fijn dat je altijd tijd had om te luisteren. Robert, ook jij was voor mij een vraagbaak en ik heb veel van je geleerd. Ik moet ook toegeven dat ik door jou nooit meer op dezelfde manier naar een woonboot zal kijken. Inge, het is alweer een tijdje geleden dat we samen op het lab stonden. Maar ik zal jou en je spekjestaart niet snel vergeten. Thomas, thanks for explaining tryptophan fluorescence to me. It was a pleasure to work with you.

Dan de mannen en vrouwen van de zesde verdieping. Robin, bedankt voor het wegwijs maken in de wondere wereld van het KcsA zuiveren. Ik hoop dat je snel weer wat zuidelijker komt wonen. Pieter, ik heb respect voor de manier waarop jij je promotie toch hebt afgerond. Ik zal je lab-optreden samen met Robbert en Vincent niet snel vergeten. Irene H, bedankt voor alle feed-back en gezellige gesprekken, tijdens proeven, pauze of zomaar in de gang. Veel geluk met de gezinsuitbreiding. Rutger, bedankt voor je gezelligheid en ik hoop dat we toch nog een keer een tochtje op de motor zullen maken.

Naast mijn directe collega's wil ik ook graag de vakgroep membraan enzymologie bedanken voor het altijd mogen lenen van enzymen en natuurlijk de onvergetelijke pink parties.

Er is meer in het leven dan werk. Dus bij deze wil ik ook graag mijn vrienden bedanken voor hun steun en interesse in de afgelopen jaren. Thijs, ik vind het jammer dat ik je zo weinig persoonlijk spreek, en dat terwijl Hamburg toch niet heel ver weg is. Nu mijn proefschrift af is ga ik proberen wat vaker die kant op te komen. Gelukkig heb je vrijwel altijd tijd om even bij te kletsen over al dan niet relevante dingen via de msn. Ik voelde me vereerd toen ik jou paranimf mocht zijn, en vind het super dat jij nu mij bij wil staan tijdens mijn verdediging.

Een mens moet ook relaxen. Marisca bedankt voor alle koppen thee, motorritjes en sauna dates. Kristel, het blijft fijn om af en toe met jou bij te kletsen en leuke dingen te doen. Ik hoop dat we volgend jaar weer wat vaker samen kunnen fietsen en thee drinken. Janneke R, zonder jou zou mijn Nederlandse samenvatting een stuk meer woorden bevatten. Thanx voor het doorlezen en je steun tijdens de rest van mijn promotie.

Verder wil ik een aantal mensen bedanken voor het faciliteren van promotie ontwijkend gedrag. Ten eerste de hellas crew (jullie weten zelf wel wie jullie zijn). Of het nou even bijlkletsen na/tijdens een training is, een kopje koffie bij de HEMA op zaterdag ochtend, een picknick bij het Strijkviertel of een fietstocht in het weekend, jullie zijn er altijd voor te porren, thanx!

Daarnaast wil ik ook Sandra H, Janneke B, Mirjam, Lydia, Stella, Mieke, Joyce. Ilse, Annemieke en Rik bedanken voor het genereren van niet promotie gerelateerde activiteiten. Het is altijd fijn om leuke mensen om je heen te hebben.

Naast vrienden heb ik ook veel steun van mijn familie ontvangen. Ik wil jullie bedanken voor de steun en interesse die jullie mij, niet alleen tijdens mijn promotie maar ook tijdens de rest van mijn leven, hebben gegeven.

Een speciaal bedankje gaat dan natuurlijk uit naar mijn ouders. Bedankt dat jullie me mijn hele leven hebben gesteund in de dingen die ik graag wilde doen. Ik weet dat het niet altijd makkelijk te begrijpen is waar ik mee bezig ben, maar dat maakt jullie steun gelukkig niet minder onvoorwaardelijk. Ik weet dat jullie trots op me zijn, maar ik ben ook trots op jullie. Bedankt voor alle steun en liefde.

Lieve Mark, zonder jou was het laatste jaar van mijn promotie nog een stuk zwaarder geweest. Ik heb bewondering voor je geduld en ben blij dat jij in het bezit bent van twee sterke armen die ik zo vaak nodig heb gehad. Jij was de stabiele factor in een voor mij zeer onzekere en stressvolle tijd. Bij jou wil ik de rest van mijn leven thuiskomen. Ik hou van je.

Heel veel liefs Erica

curriculum vitae

Erica Kremer was born on the 20th of October 1980 in Apeldoorn, the Netherlands. She graduated from the athenaeum at the Koninklijke Scholengemeenschap Apeldoorn in 1998. In September of the same year she started to Study Biology at the University of Utrecht.

This study exposed here to a broad variety of biological disciplines, including her mayor in the department of Cellular Protein Chemistry on a project involving the study of the ER (endoplasmatic reticulum)- proteins Ero1α and PDI in HEK cells, supervised by Drs. Jenny Smit and Prof. Dr Ineke Braakman. Her minor on the Redox-status of cysteines in yeast under the supervision of Dr. Jacob Winther at the Carlsberg research centre in Copenhagen gave her a feel for working in industry. In December 2004 she obtained her master degree in Biomolecular sciences at the University of Utrecht.

After a year of working outside of science in January 2006 she started her PhD project in the department of Biochemistry of Membranes under de supervision of Prof. Dr. Antoinette Killian. Here she investigated protein-lipid interactions in general and the influence of lipids on the potassium channel KcsA in specific. During her PhD she spent two weeks in the lab of Prof. Dr. Gonzalez-Ros in Elche, Spain, which allowed her to gain knowledge on the functional aspects of potassium channels. The results of her PhD project are published in this thesis.