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The potassium channel KcsA and its interaction with the lipid bilayer

I. M. Williamson, S. J. Alvis, J. M. East and A. G. Lee *

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton, SO16 7PX (United Kingdom), Fax: + 44 23 8059 4459, e-mail: agl@soton.ac.uk

Abstract. The crystal structure of the K^+ channel KcsA explains many features of ion channel function. The selectivity filter corresponds to a narrow region about 12 Å long and 3 Å wide, lined by carbonyl groups of the peptide backbone, through which a K^+ ion can only move in a dehydrated form. The selectivity filter opens into a central, water-filled cavity leading to a gating site on the intracellular side of the channel. The channel is tetrameric, each monomer containing two transmembrane α helices, M1 and M2. Helix M1 faces the lipid bilayer and helix M2 faces the central channel pore; the M2 helices participate in subunit-subunit interactions. Helices M1 and M2 in each subunit pack as a pair of antiparallel coils with a heptad repeat, but the M2 helices of neighbouring subunits show fewer interactions, crossing at an angle of about –40°. Trp residues at the ends of the transmembrane α helices form clear girdles on the two faces of the membrane, which, together with girdles of charged residues, define a hydrophobic thickness of about 37 Å for the channel. Binding constants for phosphatidylcholines to KcsA vary with fatty acyl chain length, the optimum chain length being C22. A phosphatidylcholine with this chain length gives a bilayer of thickness about 34 Å in the liquid crystalline phase, matching the hydrophobic thickness of the protein. However, a typical biological membrane has a hydrophobic thickness of about 27 Å. Thus either the transmembrane α helices of KcsA are more tilted in the native membrane than they are in the crystal structure, or the channel is under stress in the native membrane. The efficiency of hydrophobic matching between KcsA and the surrounding lipid bilayer is high over the chain length range C10–C24. The channel requires the presence of some anionic lipids for function, and fluorescence quenching studies show the presence of two classes of lipid binding site on KcsA; at one class of site (nonannular sites) anionic phospholipids bind more strongly than phosphatidylcholine, whereas at the other class of site (annular sites) phosphatidylcholines and anionic phospholipids bind with equal affinity.

Key words. Potassium channel; KcsA; lipid-protein interactions; tryptophan; reconstitution; fluorescence spectroscopy.

Introduction

It has been estimated that only 1 in 1000 billion ions hitting a square centimetre of lipid bilayer surface will pass through by simple diffusion [1]; channels are therefore required to allow ions to cross a lipid bilayer. These channels must both allow controllable passage of ions across the membrane at a high rate and be highly selective for the ions they do allow to pass. For example, for a K^+ channel the task on opening is to allow K^+ ions in, to keep Na^+ ions out and to prevent Ca^{2+} ions from getting stuck in the pore and stopping the permeation of K^+ ions. This combination of properties has been achieved in the ion channels; a voltage-gated K^+ channel allows K^+ ions to pass through at a rate of 10^6–10^8 ions s^{-1}, with an error rate of about 1 in 1000; only about 1 Na^+ ion permeates a K^+ channel for every 1000 K^+ ions that pass through [1].

The two main classes of K^+ channel are the voltage-gated or Kv family, and the Kir family. The Kv family of K^+ channels contains a conserved hydrophobic core of six transmembrane α helices and a conserved motif called the P loop that is part of the K^+ conduction pathway, located between the last two transmembrane α helices. The members of the Kir family are so called because they

* Corresponding author.

show inward rectification (the term rectification means that the channel passes current more readily in one direction than another and the term inward reflects the fact that they preferentially conduct ions into the cell). Members of the Kir family contain only two transmembrane α helices, corresponding to the last two of the helices in the Kv family, together with a P loop. Kv and Kir family K^+ channels are multimers of four subunits that make up the pore; they can also associate with a number of auxiliary subunits. Members of the Kv family of K^+ channels have structures similar to those of other voltage-gated ion channels. Na^+ and Ca^{2+} channels consist of one large polypeptide, but this contains four homologous repeats of six transmembrane α helices, S1–S6 with a P loop between helices S5 and S6. Other members of this superfamily of ion channels include the inositol 1,4,5-trisphosphate (IP_3) receptor [2] and cyclic nucleotide-gated cation channels [3]. These also exist as tetramers of six transmembrane α helical proteins, with a loop between S5 and S6 analogous to the P loop in the voltage-gated channels.

K^+ ions are important as osmoprotectants in bacteria, and bacteria also contain K^+ channels related in structure to eukaryotic K^+ channels [4]. One bacterial K^+ channel that has been overexpressed [5] and has had its structure determined [6, 7] is the KcsA channel from the Gram-positive soil bacterium *Streptomyces lividans*. It is a homotetramer, each subunit containing 160 residues with two transmembrane α helices separated by a P loop. Strong evidence that the pore structure of KcsA is similar to that of other K^+ channels has been provided by experiments in which a chimeric protein was constructed with the S5-P loop-S6 region of a Kv channel replacing the corresponding region of KcsA, giving a functional channel [8].

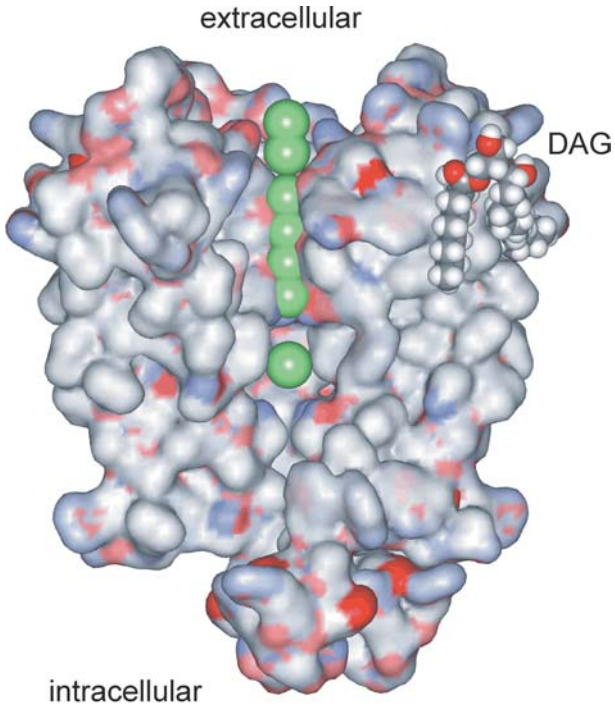

Figure 1. The structure of KcsA. Shown is a surface view from the side with the front subunit removed. The surface is coloured by atomic charge. Potassium ions are shown in green. The lipid molecule bound at the monomer-monomer interface is shown in space-fill representation (PDB file 1K4C).

Structure of the channel

The crystal structure of KcsA has been determined for the protein lacking the C-terminus (from residue 126 to 158); the N-terminal residues 1–22 and the C-terminal residues 120–126 were disordered in the crystal and so were not seen in the structure [6, 7]. The channel is a tetramer with fourfold symmetry about a central pore (fig. 1). One transmembrane α helix in each subunit (the C-terminal helix M2) faces the central pore, and the other helix (the N-terminal helix M1) faces the lipid bilayer. This is seen more clearly in a cut-away view showing only two of the monomers making up the tetrameric array (fig. 2). The inner helices are tilted with respect to the bilayer normal by about 25° and are slightly kinked so that the bundle of helices opens out on the extracellular side, but are packed more closely on the intracellular side. The result is that as described by Doyle et al. [6], the four inner helices have the appearance of an inverted tepee.

The M1 and M2 helices are organized as a pair of antiparallel coils in which each M1 helix only contacts M2 from its own subunit, whereas the M2 helices participate in subunit-subunit interactions. The residues at the M1/M2 interface show a heptad repeat typical of a coiled-coil (fig. 3). Pairing of Ala-29 on helix M1 with its corresponding residues on helix M2 (Leu-105 and Val-106) has the appearance of knobs-into-holes packing (fig. 3). However, at other positions along the helix-helix interface, residues on one helix are paired with Gly residues on the other helix (Leu-36 on helix M1 with Gly-99 on helix M2; Gly-43 on helix M1 with Val-91 in helix M2), allowing a relatively close approach of the two helices. The presence of a small residue such as Gly at the points of contact between the two helices will maximize van der Waals contact between the helices; the absence of a side chain produces a flat surface against which the side chain of other residues can pack. The lack of a side chain also means that there will be no loss of side chain entropy for the Gly residues on oligomerisation, whereas other side chains will suffer a loss of rotomeric freedom when packed at the interface. Gly is very commonly found in transmembrane (TM) α helices despite the fact that Gly is not a hydrophobic residue [9, 10].

The M2 helices cross at an angle of about –40°. The relatively steep packing angle shown by the M2 helices

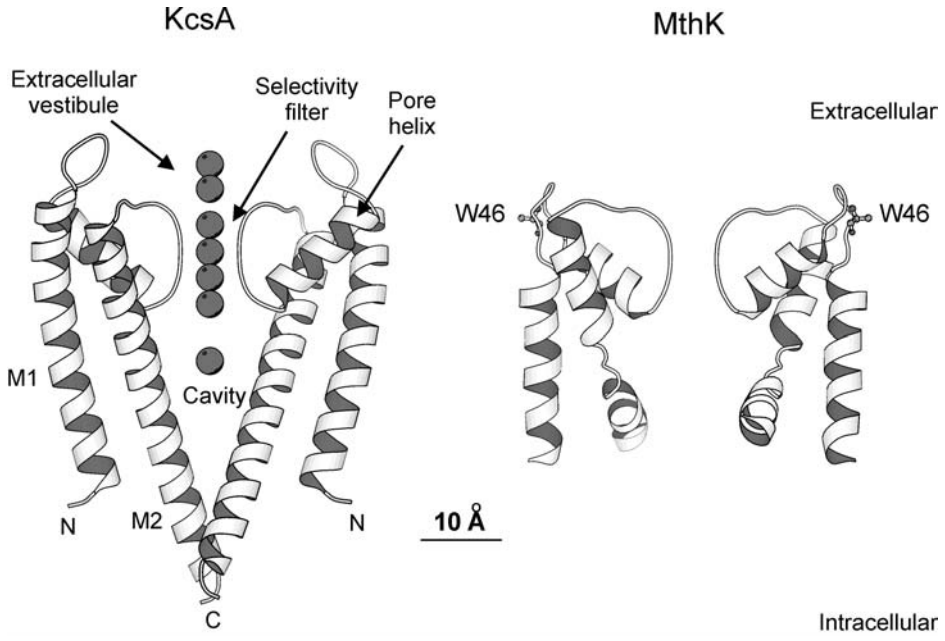

Figure 2. Cut-away view of the KcsA and MthK channels. (*Left*) Two monomers from the tetrameric structure of KcsA. The K^+ ions seen in the crystal structure are shown. (*Right*) The channel region (residues 19–98) of the MthK calcium-gated K^+ channel, believed to represent the open form of the channel [11]. Again, two monomers from the tetrameric structure are shown (PDB files 1K4C and 1LNQ).

means that the contact interface between the helices is localized to a fairly narrow region, around Ala-111 at the intracellular ends of the helices (fig. 2). It is possible that this makes any helix-helix rearrangements associated with channel gating easier, and indeed, it has been suggested that bending of the M2 helices is involved in channel opening [11], as described later.

The inner and outer helices in each monomer are connected by a stretch of about 30 residues, the P loop. The P loops of the four subunits are arranged together to form the extracellular vestibule and a narrow region referred to as the selectivity filter, which is too narrow to pass a hydrated K^+ ion (fig. 2). At this point, therefore, the K^+ ion will have to shed its waters of hydration. The selectivity filter opens out into a wide cavity of complex shape, roughly 10 Å in diameter. The size of the cavity would allow it to accommodate about 50 water molecules [1]. The cavity is connected to the cytoplasm by a somewhat featureless hydrophobic pore about 18 Å in length from which the K^+ ion can escape to the internal medium; the pore is large enough to accommodate a hydrated K^+ ion.

The selectivity filter is 12 Å long and 3 Å wide with walls constructed from backbone oxygen atoms that face into the selectivity filter. The filter contains four ion binding sites to which K^+ ions can bind in an essentially dehydrated state, surrounded by eight oxygen atoms provided by the signature sequence (^{75}TVGYG^{79}) from each of the four subunits (fig. 4). All the oxygen atoms at the three outer sites are carbonyl oxygens, whereas at the fourth site, next to the central cavity, four of the oxygens are carbonyl oxygens, the other four being side chain oxygens of Thr residues. The oxygen atoms form a stack of rings of oxygen atoms ~3–4 Å apart. The spacing of these oxygens is relatively rigid since they are part of the peptide backbone, and it is the rigidity of this part of the structure that is the basis of the mechanism of selectivity. To overcome the energy of dehydration of a K^+ ion as it enters the filter, the carbonyl oxygen atoms must take the place of

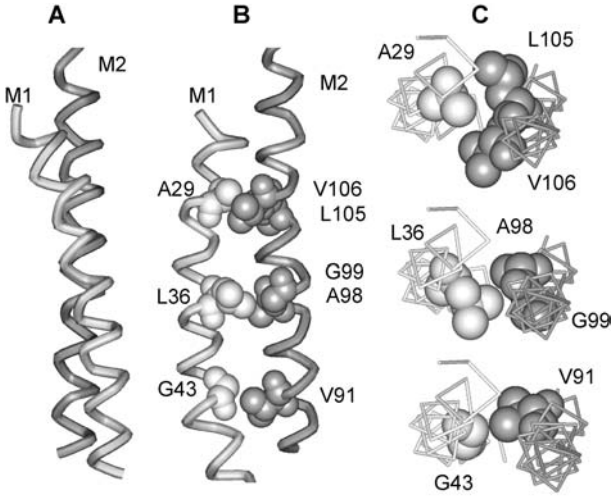

Figure 3. Coiled-coil packing of helices M1 and M2 in the potassium channel KcsA. (*A*) shows the close contact between helices M1 and M2 along their length. (*B*) shows the heptad repeat in M1 and how residues Ala-29, Leu-36 and Gly-43 contact the corresponding residues in helix M2. (*C*) shows a view down the long axes of the helices, showing the nature of the packing at the sites of close contact between the helices. Ala-29 packs as knobs into holes, but packing at the two other sites involves Gly residues, as shown.

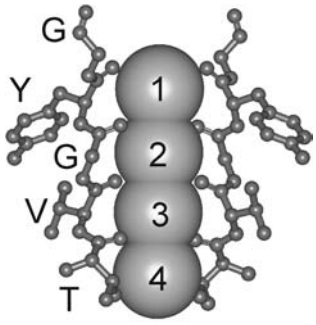

Figure 4. The selectivity filter showing the four binding sites for K^+ ions (PDB file 1K4C).

the water oxygens and come into close contact with the K^+ ion. A K^+ ion (1.3 Å radius) exactly fits into the filter, so that the losses and gains in energy on dehydration and binding are well matched. However, the rigid pore cannot collapse to allow the oxygen atoms to approach closely enough to the smaller Na^+ ion (0.9 Å radius) to compensate for the cost of dehydrating the Na^+ ion, and so the permeability of the channel to Na^+ ions is low.

The structure of the selectivity filter in the absence of K^+ is significantly different to the structure in the presence of K^+ shown in figure 4 [7]. In particular, residues Val-76 and Gly-77 at sites 3 and 2 respectively adopt different conformations in the presence and absence of K^+. In the absence of K^+ the carbonyl of Val-76 points away from the central pore and the α carbon of Gly-77 points inwards, blocking the pore. This structure for the selectivity filter must represent a nonconductive state [7]. Thus only in the presence of high K^+ concentrations does the selectivity filter adopt the structure in which it can conduct K^+ ions with high efficiency.

The movement of K^+ ions through the selectivity filter consists of hopping between successive binding sites. In molecular dynamics simulations of the channel, small 'peristaltic' changes in the filter backbone are seen to maintain optimal K–O distances as the K^+ ion moves through the filter, much as has been seen in simulations of gramicidin-cation interactions [12]. The crystal structure shows four K^+ ions within the selectivity filter; a K^+ ion is also observed in the centre of the cavity, in line with the selectivity filter (fig. 2). This ion is fully hydrated, surrounded by eight water molecules of hydration [7]. Only these eight water molecules in the inner hydration sphere of the K^+ ion are sufficiently ordered to be visible in the X-ray structure. The order of the water molecules around the K^+ ion is probably the result of many weak, indirect hydrogen-bonding interactions mediated by residues in the cavity walls. Other water molecules in the cavity are not ordered, because most of the residues lining the cavity are hydrophobic and do not provide strong hydrogen-bonding donor or acceptor groups. The consequence is that water molecules in the cavity are available to interact strongly with the K^+ ion without competition with the protein surface [7].

The K^+ ion is held in the cavity centre by the water structure around the ion. Holding the K^+ ion in this way will not decrease the rate of K^+ movement through the channel, because the rate of exchange of ordered water molecules is very fast [7]. The location of the K^+ ion in the cavity is also favoured by electrostatic factors. The segment of the P loop immediately N-terminal to the selectivity sequence is an α helix, referred to as the pore helix (fig. 2). The four pore α helices make an angle of about 45° with respect to the axis of the pore; the negative ends of the dipoles generated by the pore helices will point towards the cavity since the pore helices are oriented with their C-terminal ends pointing towards the cavity centre. The K^+ ion observed in the cavity is at the position in the cavity to which the pore helices point. The extra stabilisation energy provided for the K^+ ion in this way is important for efficient function of the channel. It has been estimated that it would cost 68 kJ mol^{-1} to move a K^+ ion from an aqueous solution to a water-filled sphere of radius 5 Å surrounded by a hydrocarbon environment, a simple model for the cavity in KcsA; the energy cost would be somewhat less for a nonspherically shaped cavity closer in shape to the cavity in KcsA [13]. Although this is much less than the energy of about 240 kJ mol^{-1} required to transfer a K^+ ion to a hydrocarbon environment [13], it still represents a significant energy barrier. Orienting the dipoles of the four pore helices towards the centre of the cavity helps to minimize this energy barrier [13]. An additional effect of the pore dipoles is to stabilize monovalent ions at the centre of the membrane but not divalent ions [13]. This is important in explaining selectivity. The selectivity filter is located at the extracellular end of the pore. Thus cations other than K^+ can probably enter the pore from the intracellular side and penetrate two-thirds of the way across the membrane, potentially blocking the pore at the selectivity filter. However, the selectivity of the cavity for monovalent ions over divalent ions such as Mg^{2+} present in the intracellular environment will favour monovalent ions. The much higher concentration of K^+ present in the cytoplasm compared with Na^+ will ensure that the monovalent cation in the cavity will be mainly K^+.

Apart from the selectivity filter, the pore lining is mostly hydrophobic. This is consistent with the observation that hydrophobic cations bind in the pore of the K^+ channel [14]. Figure 5 shows a tetrabutylammonium ion bound in the central cavity, blocking internal entry to the selectivity filter [15]. It has been suggested by Doyle et al. [6] that the hydrophobic lining to the majority of the pore may facilitate the flow of K^+ ions by minimizing their interaction with the residues lining the pore: if the channel lining were polar, interaction between a K^+ ion and the channel lining would slow movement of the ion. The de-

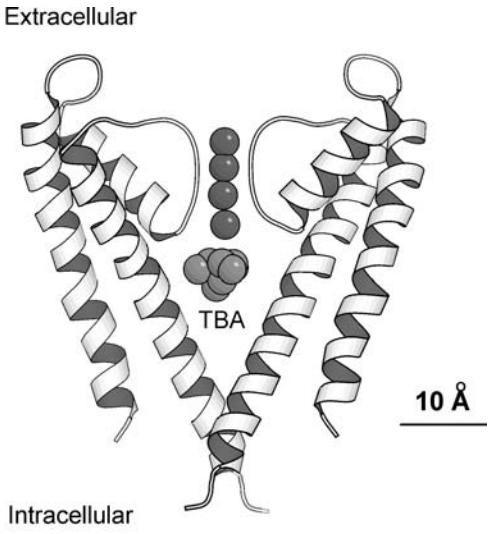

Figure 5. The binding site for tetraalkylammonium salts in KcsA. A cross-section of KcsA is shown with four K^+ ions in the selectivity filter and a tetrabutylammonium (TBA) ion in the central cavity (PDB file 1J95).

sign of the inner pore and cavity ensures a low resistance pathway for movement of an ion from the selectivity filter. The rate limiting step in K^+ movement therefore occurs over the short length (12 Å) of the selectivity filter. The location of the large water-filled cavity in the centre of the membrane has reduced the barrier to diffusion of a K^+ ion from the hydrophobic thickness of a lipid bilayer (30 Å) to just 12 Å.

Under physiological conditions the selectivity filter normally contains two K^+ ions, and this dual occupancy is important for the function of the channel [16]. A single K^+ ion would be held very tightly in the filter. This would ensure selectivity of the channel for K^+ ions but would give a slow rate of transfer through the channel. However, the presence of a second K^+ ion results in mutual repulsion between the ions, which will balance the attractive force between the K^+ ion and its site. The driving force for movement through the channel is therefore the coordinated movement of the two ions [16]. The pair of K^+ ions occupies either the 1 and 3 or 2 and 4 positions in the filter (fig. 4), separated by a single water molecule. The ions move backwards and forwards between these pairs of positions until a third K^+ ion enters on one side of the filter, causing displacement of a K^+ ion on the opposite side. The design of the filter is such that the energy difference between occupation of the 1 and 3 sites and occupation of the 2 and 4 sites is small, ensuring a low activation barrier for movement of K^+ ions through the channel [16].

Negatively charged residues at the cytoplasmic mouth of KcsA attract cations, effectively concentrating them around the mouth the channel. Asp-80 is located immediately above the selectivity filter (on the extracellular side). It is solvent exposed and thought to be present in the ionised form [17, 18]. Its negative charge will help to attract K^+ ions to the extracellular vestibule. Located in the pore helix close to the selectivity filter is Glu-71. Glu-71 is buried in a protein cavity and is believed to be uncharged in the absence of K^+ but charged in the presence of K^+ [17, 18]. Although negative charge on Glu-71 could, in principle, form part of an attractive electrostatic potential for cations passing through the pore, calculations show that the carbonyl groups in the selectivity group provide sufficient stabilization [17]. Further, Glu-71 is not conserved in the family of potassium channels and so cannot be an essential residue.

The net result of all these structural features is that KcsA is permeable to K^+ and the close analogues Rb^+, NH_4^+ and Tl^+ but is impermeable to Na^+, Li^+ and Cs^+ [19]. Selectivity to K^+ over Na^+ is close to 10^4, and the rate of ion movement of K^+ through the channel is close to the diffusion-limited rate of 10^8 ions per second [20]. Ion movement from the cytoplasm has been summarised by Clapham [1] as follows. The concentrations of Na^+ and K^+ in the cytoplasm are about 1–10 mM and 140 mM, respectively, with about 30 mM Cl^-. These ions will be moving at about 1 Å ps^{-1}, with an average separation between K^+ ions of about 200 Å. The negative charges near the intracellular mouth of the K^+ channel will attract K^+ ions to the mouth. If the channel is open, K^+ and also, presumably, Na^+ ions will enter the channel and move through the inner pore to the cavity. An ion in the cavity will be surrounded by water molecules and the negative dipoles of the four pore helices will also stabilize the ion and prevent the ion moving out again into the cytoplasm. The K^+ ions can now exchange their waters of hydration for carbonyl oxygens of the selectivity filter; water molecules substitute around a K^+ ion with a rate of 1 ns^{-1}. The smaller Na^+ ion cannot shed its more tightly packed water molecules and so does not move into the filter. Entry of a K^+ ion into the filter pushes out of the filter the outer of the two K^+ ions already in the filter. On exiting the filter, a K^+ ion will rehydrate and diffuse out into the bulk solution. The design of the channel is such that the limiting diffusion step has been reduced to only 12 Å.

Channel opening

The open probability of the KcsA channel increases as the intracellular pH decreases; electrophysiological recordings show that the pH-sensitive side of the channel is the internal side [21, 22]. This is unexpected, since under most conditions the intracellular pH is tightly regulated. It is possible therefore that the natural 'ligand' controlling opening of KcsA is not H^+ but some other unidentified activator protein or ligand [23]. The gating site is likely to be located at the point where the four M2 helices cross on the intracellular side, since at this point the pore

is very narrow (about 4 Å diameter; see fig. 1) and is lined with hydrophobic residues. If this is correct then the crystal structure for KcsA would correspond to the closed form of the channel. Experiments in which Cys residues have been introduced into the pore of the KcsA channel and labelled with nitroxide free radicals suggest that the M2 helices could rotate and move at low pH, leading to a small increase in diameter at the point where the M2 helices cross [24]. However, the observation that tetrabutylammonium ions with a diameter of ~10–12 Å can enter the central cavity suggests that in the open form the pore must be very wide [11]. The argument has been taken further by comparing the structure of KcsA with the structure of the calcium-gated K^+ channel MthK, which has been determined at 3.3 Å resolution, sufficient to resolve the polypeptide backbone but not the positions of the side chains [11, 25]. As shown in figure 2, the structure of the selectivity filter in MthK is very similar to that in KcsA, but the arrangement of the transmembrane α helices on the intracellular side is very different, particularly for M2. Whereas in KcsA the M2 helices are almost straight and cross on the intracellular side, in MthK the M2 helices are bent, leaving a large pore of diameter at its smallest part of about 12 Å leading to the inner surface of the selectivity filter so that the central cavity is essentially continuous with the aqueous medium on the intracellular side of the membrane (fig. 2). The bend in the M2 helices occurs at a hinge point corresponding to a conserved Gly residue, Gly-83 in MthK and Gly-99 in KcsA (fig. 3; [11]). This Gly, referred to by Jiang et al. [11] as the gating hinge, is conserved in the superfamily of K^+ channels. Gly is often found at locations where flexibility in a protein is required, since Gly can adopt a wide range of main-chain dihedral angles. Also conserved is an Ala or Gly residue, five residues C-terminal of the gating hinge. The side chain of the amino acid at this position points in to the centre of the open pore, at its narrowest position. A small residue at this position is therefore important to ensure the maximum diameter for the open pore.

Channel opening requires an outward-directed force on the C-terminal end of M2 to move the C-terminal end about the gating hinge [11]. The C-terminal ends of M2 are connected to the intracellular ligand binding domain in this superfamily of ion channels, the ligand binding domain being different for each class of channel. Ligand binding to the ligand binding domain presumably leads to a conformational change in the domain able to exert the required force on M2.

Possible roles for the N- and C-terminal regions of KcsA, not visible in the crystal structure, in the gating process have to be considered. It has been suggested, based on site-directed spin labelling, that the N-terminus of KcsA is an interfacial α helix pointing away from the core of the channel and that the C-terminal ends of the four subunits form a helical bundle extending some 40–50 Å into the cytoplasm [23]. This would be rather similar to the structure of the mechanosensitive channel McsL, where the C-terminal region also forms a helical bundle, although, in this case, it is a five-helix bundle [26]. The fact that the N-terminal region appears to point away from the channel pore suggests that the N-terminal region may not be important in the function of KcsA. Deletion of the first 20 residues in KcsA leads to dramatic reduction in levels of expression, suggesting that the N-terminal region contains signalling information important for correct folding and targeting [23]. Proteolytic removal of the C-terminal region (residues 125–160) leads to a reduction in stability for the KcsA tetramer and an increase in ion flux through the channel at neutral pH, suggesting a role for the C-terminal region in stabilizing the closed state [27]. However, KcsA with the C-terminal domain removed does still gate in a pH-sensitive manner, suggesting that the pH sensor could be a stretch of charged residues (Arg-117, Glu-118 and Glu-120) at the end of M2 [23].

KcsA in the lipid bilayer

KcsA must be tightly packed into the surrounding lipid bilayer to prevent packing defects at the protein-lipid interface through which ions could move. Trp residues are often found at the ends of transmembrane α helices and have been suggested to serve to 'anchor' the helices into the membrane [28]. The Trp residues in KcsA form clear girdles at the two faces of the lipid bilayer, the rings of the Trp residues being almost parallel to the surface of the membrane (fig. 6). On the extracellular side of the membrane, Tyr residues also form a clear girdle 'above' the band formed by the Trp residues. Above and below the girdles of aromatic residues on the two sides of the membrane are girdles of charged residues, Arg-52, Arg-64, Arg-89 and Glu-51 on the extracellular side and Arg-27, Arg-117, Arg-121, Glu-118 and Glu-120 on the intracellular side. These residues presumably provide charged residues required for good interaction with the lipid headgroup region of the bilayer. Of the five Trp residues in KcsA, Trp-26 and Trp-113, at the intracellular ends of transmembrane α helices M1 and M2, respectively, are exposed to the lipid bilayer. At the extracellular end of M2, Trp-87 is also exposed to the lipid bilayer, but Trp-67 and Trp-68 are located away from the lipid-protein interface as part of the short pore helix that points into the intracellular cavity. It has been suggested that aromatic residues around the selectivity filter (Trp-67, Trp-68, Tyr-78 and Tyr-82) form a cuff around the pore helping to keep the opening taut [6].

Two partial lipid molecules are seen in the X-ray structure (fig. 6), one modelled as nonan-1-ol and the other as a diacylglycerol with one C14 and one C9 chain [7]. Purified KcsA contains ~0.7 phosphatidylglycerol molecules per

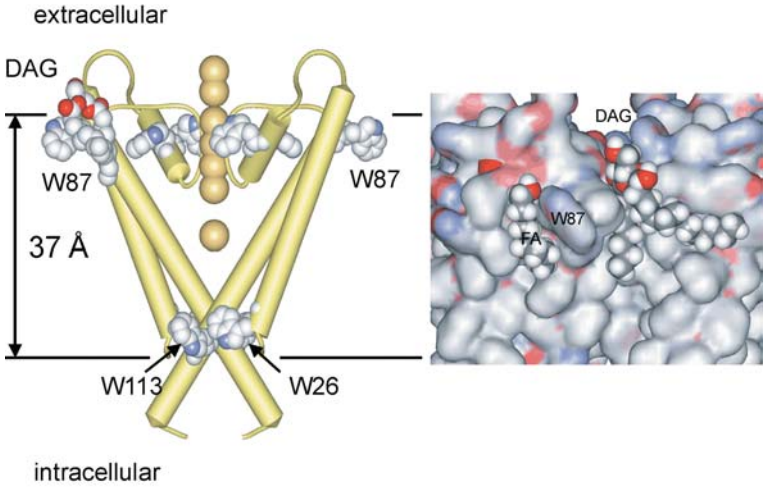

Figure 6. Lipid binding to the KcsA channel. (*Left*) For clarity, only two monomers from the tetrameric structure are shown. Trp residues are shown in space-fill representation. The lipid molecule modelled as a diacylglycerol (DAG) is also shown in space-fill representation. Also shown are the positions of the K^+ ions. (*Right*) The surface of the KcsA channel on the extracellular side of the membrane, showing the DAG molecule and a fatty acyl chain (FA) binding to clefts on the protein surface close to Trp-87 (PDP file 1K4C).

KcsA monomer so that the molecule modelled as a diacylglycerol is probably a phosphatidylglycerol whose headgroup is too disordered to be resolved [30]. The diacylglycerol moiety of the lipid binds into a groove on the surface close to Trp-87, between two KcsA monomers. The C9 chain is located in a groove between the pore helix of one monomer and the transmembrane α helix M2 of the adjacent monomer. The C14 chain is located more peripherally on the surface of the pore helix and the transmembrane α helix M1 of the same monomer. The single chain modelled as nonan-1-ol is located in a groove between transmembrane α helices M1 and M2 of a single monomer, and could correspond to the alkyl chain of the detergent dodecylmaltoside (fig. 6). The anionic headgroup of the phosphatidylglycerol molecule probably interacts with Arg-64 and Arg-89 located in the girdle of charged residues above Trp-87.

The phosphatidylglycerol molecules are bound in what we have referred to as nonannular sites, to distinguish them from the boundary or annular sites that make up the lipid-exposed external surface of the protein [31]. In fact, although only a few crystal structures of membrane proteins show resolved lipid molecules, many of these lipid molecules are bound to nonannular sites between transmembrane helices, as found in KcsA; examples include cardiolipin bound to the bacterial photosynthetic reaction centre and cardiolipin and other lipid molecules bound to cytochrome c oxidase and cytochrome bc_1 (see e. g. [32]). These lipid molecules are often essential for activity, cardiolipin, for example, being required for activity of many of the proteins important in bioenergetics.

As shown in figure 6, the diacylglycerol molecule is located with the Trp ring system of Trp-87 just below the glycerol backbone of the diacylglycerol. This is consistent with electron spin resonance (ESR) studies of KcsA in lipid bilayers that suggest that Trp-87 is located close to the glycerol backbone region of the lipid bilayer [33]. Trp-113 on the intracellular side has also been suggested on the basis of ESR results to be located within the hydrocarbon region of the bilayer [34]. If, like Trp-87, Trp-113 is located just below the glycerol backbone region, the thickness of the hydrocarbon core of the lipid bilayer would be about 37 Å.

Binding constants for phosphatidylcholines to KcsA depend on fatty acyl chain length [35], as shown in figure 7. Binding constants increase with increasing chain length from C10 to C22, with a small decrease from C22 to C24. This contrasts with the lipid binding constants for the α barrel porin OmpF (fig. 7), for which the optimal chain length is C14, corresponding well to the average chain length in the bacterial outer membrane where OmpF is located [36]. It is interesting, therefore, that the optimal chain length for lipid binding to KcsA is C22, since phosphatidylcholines with this chain length give a bilayer of hydrophobic thickness about 34 Å, matching the estimated hydrophobic thickness of KcsA (fig. 6).

The fatty acyl chains of *Streptomyces*, the bacterium from which KcsA is derived, are unusual in being mostly branched-chain saturated C14, C15 and C16 iso-acids and C15 anteiso-acids [37]. The thicknesses of bilayers of branched-chain lipids appear not to have been determined and may be different from those of the normal unsaturated phospholipids. However, if the thickness of the lipid bilayer in *Streptomyces* is comparable to that in a bilayer of di(C16:1)PC, then the hydrophobic thickness of the bilayer would be significantly less than that giving optimal binding to KcsA. Thus, the helices in KcsA may be in a state of slight stress in the native membrane, or

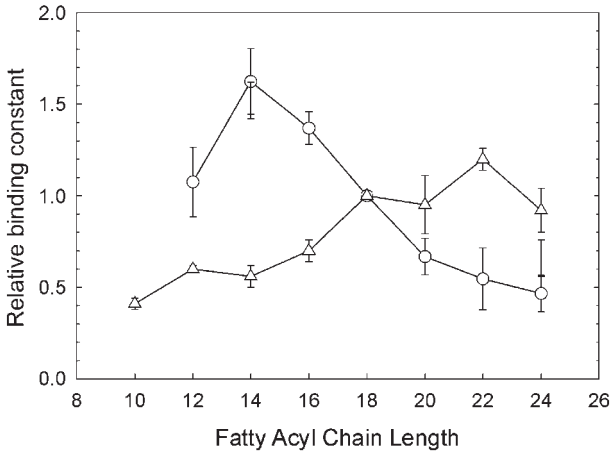

Figure 7. Relative lipid binding constants for OmpF and KcsA. The binding constants of OmpF (○) and KcsA (△) for phosphatidylcholines relative to that for di(C18:1)PC are plotted as a function of fatty acyl chain length. All lipids are in the liquid crystalline phase. Data from [35, 36].

the tilt of the transmembrane α helices for KcsA in the membrane may be different from those in the crystal structure.

The efficiency of hydrophobic matching between KcsA and its surrounding lipid bilayer has been demonstrated from observations of the fluorescence emission spectra of the Trp residues in KcsA [35]. Fluorescence emission spectra of Trp residues are environmentally sensitive [38], and any major change in the location of the Trp groups in KcsA relative to the lipid bilayer with changing bilayer thickness would be reflected in major changes in fluorescence emission spectra. In fact, observed changes in Trp emission spectra with changing bilayer thickness in the chain length range C10–C24 are very small, suggesting that the Trp residues at the ends of the transmembrane α helices maintain their positions close to the glycerol backbone region of the bilayer [35]. The most likely changes in KcsA to achieve hydrophobic matching with the surrounding lipid bilayer are a tilt of the transmembrane α helices or a rotation of the Trp residues at the ends of the transmembrane α helices about the C_α–C_β bond linking them to the helix backbone. The relatively steep packing angle of the TM2 helices means that the contact interface between the helices is localized to a fairly narrow region, making helix-helix rearrangements relatively easy. A simple geometrical calculation shows the required magnitude of tilting if hydrophobic matching followed just from a change in tilt angle. The helices in the crystal structure of KcsA are tilted at about 25° with respect to the normal to the membrane. Assuming that the crystal structure of KcsA corresponds to the structure that would be adopted in a bilayer of thickness 37 Å, the length of the transmembrane α helices would be 40.6 Å (37/sin65). The angle of tilt of the helices with respect to the bilayer normal will have to increase to about 43°, 50° and 56° to match the thicknesses of bilayers of di(C18:1)PC, di(C16:1)PC and di(C14:1)PC, respectively.

Lipid headgroup structure is also important for the proper function of KcsA. Studies with KcsA reconstituted into liposomes showed that a functional channel could only be obtained in the presence of anionic phospholipid, with no specificity for which anionic lipid, which could, for example, be phosphatidylglycerol, phosphatidylserine or cardiolipin [30, 39]. Anionic lipid is not required for formation of the KcsA tetramer, since tetramers are formed when KcsA is reconstituted into bilayers of phosphatidylcholine in the absence of anionic lipid [30, 35]. Instead, it has been suggested that the presence of the anionic lipid 'cofactor' bound between the transmembrane α helices could be important in the gating process, because opening and closing the gate must involve movement of the transmembrane α helices [30]. Fluorescence quenching studies show that whereas anionic phospholipids and phosphatidylcholines bind with similar affinities to the annular binding sites around KcsA, anionic phospholipids bind with high affinity to the nonannular sites from which phosphatidylcholines are either excluded or for which they have a very low affinity [J. M. Williamson et al. unpublished studies].

Patch-clamp studies of K_{ATP} channels, members of the Kir family whose opening is blocked by intracellular ATP, have shown them to be modified by anionic phospholipids such as phosphatidylserine and the phosphatidylinositols [40, 41]. The presence of PtdIns(4,5)P_2 increased the half-maximal inhibitory concentration of ATP from about 10 μM to more than 3 mM, so making the channels effectively insensitive to ATP [42, 43]. The K_{ATP} channel is an octameric complex of Kir6.2 and SUR subunits, where the Kir6.2 subunits form the ATP-sensitive pore and the SUR subunits (sulphonylurea receptor) make the channel sensitive to channel regulators such as sulphonylurea drugs and K^+ channel openers. PtdIns(4,5)P_2 binds directly to the Kir channel, to the C-terminal region [41, 42]. Mutation of a pair of Arg residues in the C-terminal region leads to a reduced sensitivity to PtdIns(4,5)P_2 and other anionic lipids [40–42, 44]; two other positively charged binding sites for PtdIns(4,5)P_2 have also been identified in the C-terminal region of Kir2.1 [45]. The degree of activation is related to the number of negative charges in the lipid headgroup, and the effect of anionic lipids can be reduced by addition of polyvalent cations able to screen the negative charge [40]. The mechanism of the effect is not known, but one possibility is that binding of the C-terminal region of the channel to anionic phospholipids in the membrane leads to changes in the structure of the ATP binding site, reducing its affinity for ATP [46]. Since they involve the C-terminal region of the channel, these binding sites for anionic phospholipid must be unrelated to the binding site for anionic lipid

identified on KcsA, located on the extracellular side of the membrane. The epithelial sodium channel EnaC is also modulated by anionic phospholipids, but this time through binding to the N-terminal region of the channel [47].

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