Chemical basis for alkali cation selectivity in potassium-channel proteins

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The determination of the crystal structure of a K^+-selective channel protein from *Streptomyces lividans* reveals how the rapid movement of K^+ across membranes is catalyzed by a large family of pore-forming proteins. Many features of the structure mirror hypotheses, predictions and models of K^+ channels developed over the past four decades of functional analysis.

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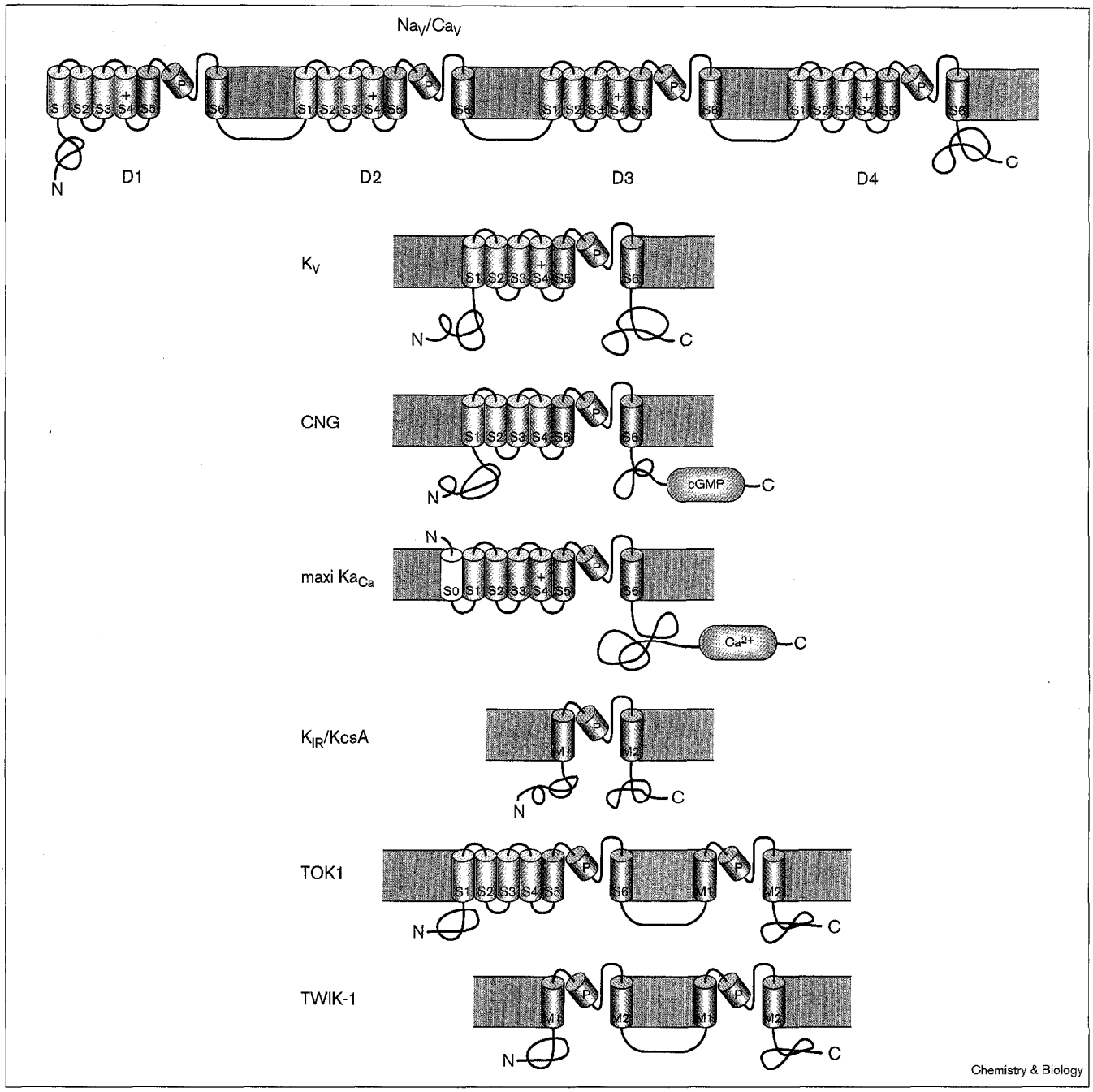
Introduction

The recent publication by Doyle *et al.* [1] of the crystal structure of a K^+ channel protein from *Streptomyces lividans* (KcsA) is a signal achievement in membrane biochemistry and molecular physiology. The work revealed the first actual molecular structure of a member of a family of membrane proteins (P-region channel proteins) that mediate the ion-selective, electrodiffusive movement of inorganic cations across cell membranes. This superfamily of ion-channel proteins includes K^+ channels, Na^+ channels, Ca^{2+} channels and cation-selective cyclic nucleotide-gated channels (Figure 1). These proteins are crucial circuit elements for electrical signaling in the nervous systems of sentient species like ourselves and effectors of a host of related physiological processes, such as ionic homeostasis, muscle contraction, neurotransmitter and hormone secretion, and sensory perception. In fact, all cellular life forms, sentient or otherwise, including plants, yeast, and archeon microbes, have homologs of P-region proteins that are structurally related to KcsA [2]. Aside from defining a new fold for membrane channel proteins, the KcsA molecule reveals the chemical basis for K^+-selectivity of such channels. As no other highly K^+-selective channel protein has yet been described that is unrelated to the P-region family, it seems likely that, in looking at the KcsA structure, we are gazing at the essential kind of molecular device that all earth-based life uses to endow lipid membranes with selective permeability to K^+. To grasp the significance of KcsA, one has to understand how admirably this structure fulfills the expectations of channel-ologists who strove to envision it. Rather than reiterate all of the structural details presented in the original analysis [1], this perspective will replay part of the drama that led up to KcsA, a story of intensive investigation that attempted to learn how the structure of a K^+ channel should look, in advance of actually seeing it.

The ghost of black box biophysics

As far back as the days when Luigi Galvani (1737–1798) observed that partially dissected frog legs can be made to contract by connecting them to voltage sources such as lightning, many researchers have found something electrifying about excitable cell membranes. It took generations of biophysicists, however, to work out the ionic basis of the resting membrane potential, the mechanism of nerve action potentials, and the unitary properties of ion channels before the remarkable physical chemistry performed by K^+-selective channels became clear. The modern study of ion-channel function is facilitated by electrophysiological techniques such as patch clamping,

Figure 1

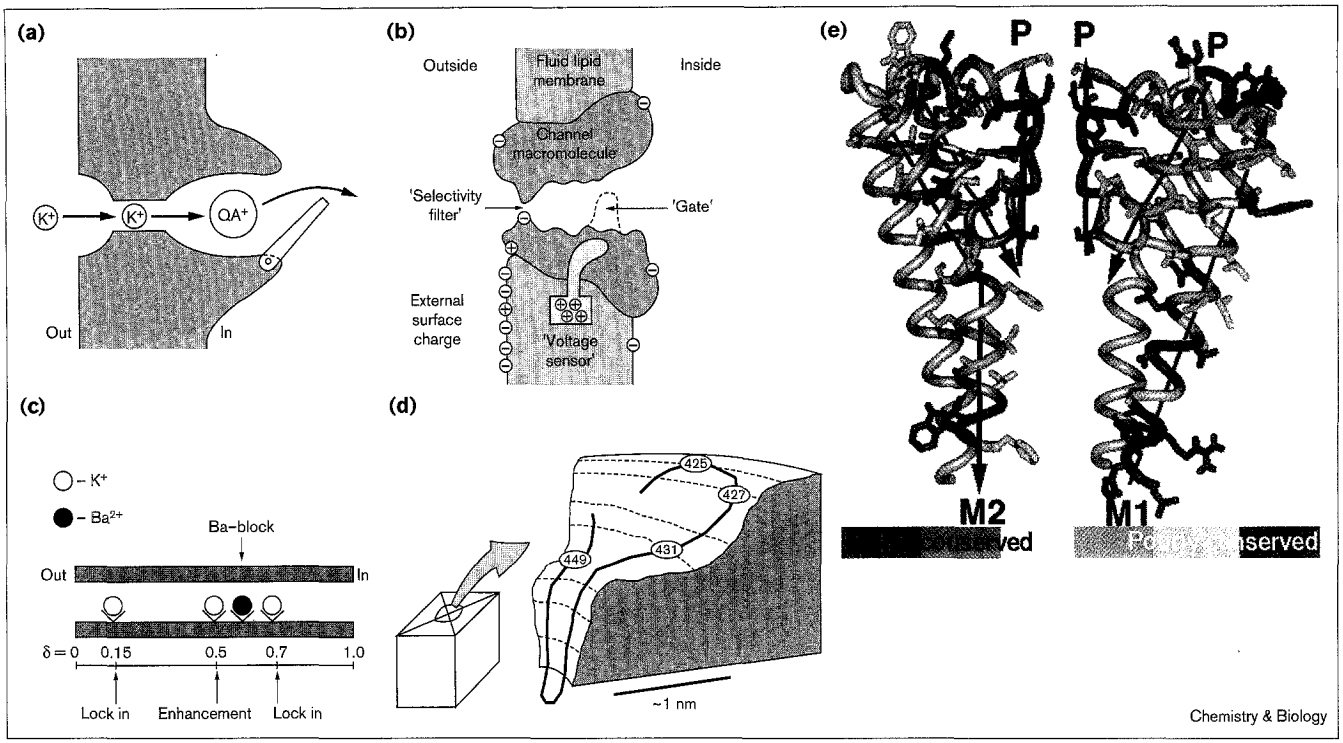


Superfamily of P-region channel proteins. Major classes of ion-channel proteins that contain a pore-forming domain homologous to the M1–P–M2 domain of KcsA. Predicted membrane topology folds are illustrated for voltage-gated Na^+ channels (Na_V), voltage-gated Ca^{2+} channels (Ca_V), cyclic nucleotide gated channels (CNG), large conductance Ca^{2+}-activated K^+ channels (maxi K_{Ca}), inward rectifier K^+ channels (K_{IR}) and KcsA, two-pore outward rectifier K^+ channel from yeast (TOK1), two-pore inward rectifier K^+ channel (TWIK-1). The K^+ pore-forming domain corresponding to the KcsA structure is pink.

which can measure channel-mediated ionic currents with high temporal resolution down to the level of a solitary channel molecule. Single-channel measurements show that various types of highly K^+-selective channels have unitary conductances in the range of 10–300 pS, which corresponds to $6 \times 10^6 - 2 \times 10^8$ ions moving through the channel per second at 100 mV of driving force.

These high transport rates (consistent with transmembrane diffusion of ions through a pore of atomic dimensions)

Figure 2



Some proposed models of P-region channel proteins. **(a)** Blocking studies of squid axon K_V channels by quaternary alkylammonium (QA^+) cations led Armstrong [10] to propose a narrow pore with wide outer and inner vestibules and an inner gate that can trap organic cations or prevent their entry. The cartoon diagram is fashioned after Hille [4]. **(b)** Biophysical studies of Na_V channels by Hille [47] suggested a pore architecture surprisingly similar to K^+ channels that included a short, narrow selectivity filter and a large internal vestibule that can bind hydrophobic local anesthetic drugs, such as the tertiary amine lidocaine. The cartoon is from Hille [47]. **(c)** Single-channel analysis of the kinetics of Ba^{2+} block of maxi K_{Ca} channels by Neyton and Miller [8] provided strong evidence for multiple binding sites for K^+ and Ba^{2+} and multi-ion occupancy of the single-filing region. The diagram is from Neyton and Miller [8]. **(d)** A simple model of the relative location of residues in the P-region of *Shaker* K_V channels that interact with charybdotoxin and related scorpion toxins in the outer vestibule as described by Miller [16]. **(e)** Predicted backbone structure of two monomers of the tetrameric *S. lividans* KcsA channel based on K^+ channel mutagenesis data and principles of membrane protein modeling. This figure, based on a model of KcsA by Guy and Durell [40], was kindly provided by H. Robert Guy. Residues are colored according to the degree of relative conservation in multiple alignments of many K^+ channel proteins.

would not be so extraordinary except for the fact that K^+ channel pores allow only the following few inorganic cations to carry current at a measurable rate: Tl^+, Rb^+, K^+, NH_4^+ and, in some cases, Cs^+. As larger cations such as $CH_3NH_3^+$ are impermeant, the narrowest cross-section of the pore cannot be much larger than the ionic diameter of Cs^+ or ~3.4 Å [3]. K^+ channel pores must also incorporate a very efficient mechanism for excluding the smaller alkali cations Li^+ and Na^+ that have ionic diameters of ~1.2 Å and ~1.9 Å, respectively. On the basis of such ionic selectivity measurements, biophysicists inferred that K^+ channel proteins should contain a specialized molecular structure called a 'selectivity filter' [4] (Figure 2b). This term refers to a narrow region of the pore that is ultimately responsible for size-selective filtration and chemical discrimination of a specific group of preferred inorganic cations that fall within the molecular cutoff limit.

More detailed biophysical studies of the ion-permeation mechanism identified other striking features of K^+ channel pores. The conduction pathway must be capable of binding more than one K^+ ion simultaneously and at least part of the tunnel must have a single-filing region (i.e. a region where several K^+ ions and H_2O molecules are constrained to move in a unimolecular chain without passing each other). The original evidence for multi-ion occupancy of K^+ channels is based on measurements of the Ussing flux ratio exponent, n. This parameter is derived from slope of the logarithm of the ratio of unidirectional influx and efflux of K^+ plotted against the driving force (membrane voltage minus the Nernst potential). It is measured by radiotracer experiments in a voltage-clamped membrane. For a mechanism involving independent electrodiffusion of ions, n should equal 1.0. Values of n in the range of 2.5–3.4 measured for various K^+ channels signify that the movement of three or more K^+

ions in the same direction is electrically coupled [5,6]. Other strong lines of evidence for multi-ion occupancy are based on observations of inhibition or block of K^+ channel current by poorly conductive ions such as Cs^+ and Ba^{2+}. Numerous equilibrium and kinetic studies of ion-blocking behavior can only be reconciled in a pleasant manner by postulating that the pore can be simultaneously occupied by more than one K^+, Cs^+ or Ba^{2+} ion [4,7]. A particularly incisive analysis of Ba^{2+} block of a high-conductance K^+ channel (a Ca^{2+}-activated K^+ channel) provided evidence for four linearly arranged binding sites for K^+/Ba^{2+} in the conduction pathway [8] (Figure 2c). Under particular conditions, some of these sites exhibit micromolar affinity for K^+ and Ba^{2+}. A binding affinity of this magnitude suggests that there must be some sort of tight coordination of K^+ and Ba^{2+} by ligand groups of the protein.

One of the most intriguing mysteries pondered for K^+ channels is: how do these proteins solve the electrostatic problem of transferring a strongly hydrated K^+ ion from an aqueous solution of high dielectric constant ($\varepsilon_{H_2O} = 80$) across a hydrophobic membrane composed of hydrocarbon with a low dielectric constant ($\varepsilon_{alkane} \approx 2$) and low polarizability? Model electrostatic calculations indicate that the energetic cost of such a process is enormous, so the channel protein must provide a mechanism for lowering this energy barrier [4,9]. Before the determination of the KcsA structure, the accepted wisdom was that suitable chemical groups (–OH, –C=O, $–COO^-$) probably line the wall of the narrowest portion of the channel to substitute for oxygen atoms of H_2O in energetically stabilizing the partially dehydrated K^+ ions. In this spirit of inquiry, images of the probable structures of channel proteins first appeared as cartoon-like diagrams (Figure 2a–c), ghosts derived from the black box approach of pure biophysics. This approach sustained itself by making sophisticated electrophysiological measurements and fitting quasi-realistic physical models to the data in order to deduce intimate features of an unseen entity. Despite the naive simplicity of many of the physical models used to analyze K^+ channel behavior, numerous deductions that ultimately proved to be accurate are a strong testament to the power of this approach.

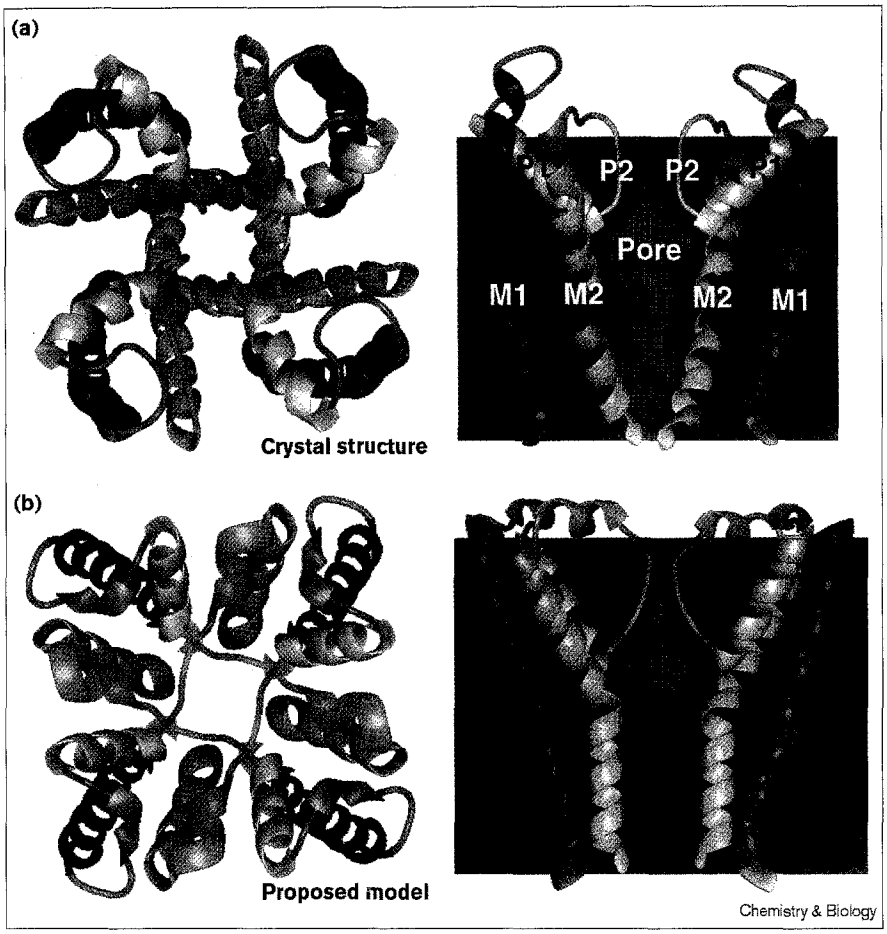
Is it pharmacology, TEA^+ time or pick your poison?

A sharper silhouette of the K^+ channel emerged from the darkness by exposing the channel to various organic molecules. In particular, using the alkyl derivatives of the ammonium ion, such as tetraethylammonium (TEA^+), proved to be most informative. Alkylammonium ions physically block the K^+ channel pore by binding transiently in the same pathway used by permeating ions. By varying the chain length of *n*-alkyl groups attached to ammonium and studying how this affects blocking activity, researchers deduced a number of key structural and chemical features of the ion-conduction pathway. An especially important finding was that large hydrophobic derivatives of TEA^+ are more potent blockers from the cytoplasmic (internal) side of K^+ channels [10]. For example, tetrapentylammonium ($TPeA^+$) blocks the squid axon K^+ channel with a K_D of 39 μM from the internal side compared with a K_D of 360 μM for internal TEA^+ [11]. Likewise, the long-chain TEA^+-derivative, decyltriethylammonium (C_{10}-TEA^+), blocks *Shaker*, a cloned K^+ channel, with a K_D of 0.75 μM from the inside compared with 390 μM for TEA^+ [12]. Systematic studies of the binding energy of such TEA^+ derivatives are consistent with a hydrophobic interaction, indicating that at least part of the lining of the inner mouth of K^+ channels must have a nonpolar surface.

Another interesting phenomenon is that cations as large as TEA^+ and C_{10}-TEA^+ can actually be physically trapped inside the inner vestibule of some voltage-activated K^+ channels by a closing process that involves a conformational change associated with channel gating [10,13]. Correspondingly, K^+ channels that are in the closed state of activation gating cannot be blocked by internal TEA^+ until they are gated open (Figure 2a). Measurements of the voltage dependence of block by various TEA derivatives indicates that the internal binding site for alkylammonium cations senses ~20% of the transmembrane electric field [12]. (According to the Woodhull model [14] of voltage-dependent block, the apparent binding affinity of a cationic blocker on the internal side is enhanced by a positive inside membrane potential, which drives the blocker inside the pore.) In contrast, block by external TEA^+ is essentially voltage-independent and is not responsive to increased hydrophobicity of the blocking molecule in the same manner as the internal blocking site. Some K^+ channels are strongly blocked by 1000 μM external TEA^+, whereas others are quite insensitive to external TEA^+. Such observations suggested that the external TEA^+-binding site has a relatively polar surface, is located outside the transmembrane electric field and contains variable molecular determinant(s) that modulate the binding energy for TEA^+. By taking readings of TEA^+, researchers in the K^+ channel field dreamed up the following picture. The innermost end of the channel contains a gate that opens and closes the pore to K^+ ions and also controls access to some relatively large organic cations (diameter of TEA^+ = 8.2 Å). Beyond this gate on the inside, there must be some kind of large cavernous region with hydrophobic walls that can apparently sequester blocking molecules as large as $TPeA^+$ (diameter ≈ 12 Å) and as long as C_{10}-TEA^+ (length ≈ 14 Å). Beyond this must lie a narrow selectivity filter (diameter ≈ 3.3 Å) that whizzes K^+ through but bounces out most other ions. This narrow filter finally opens at the outside of the membrane to a shallow, more polar vestibule that contains another site for TEA^+.

Figure 3

Ribbon diagram of the KcsA structure compared to a proposed model. The top left figure is a diagram of the crystal structure of the KcsA tetramer as viewed from the extracellular surface. The top right figure is a side view of two monomers of KcsA showing an outline of the internal pore and cavity. The bottom figures are corresponding views of a proposed model of KcsA by Durell *et al.* [40]. This figure was kindly prepared by H. Robert Guy.

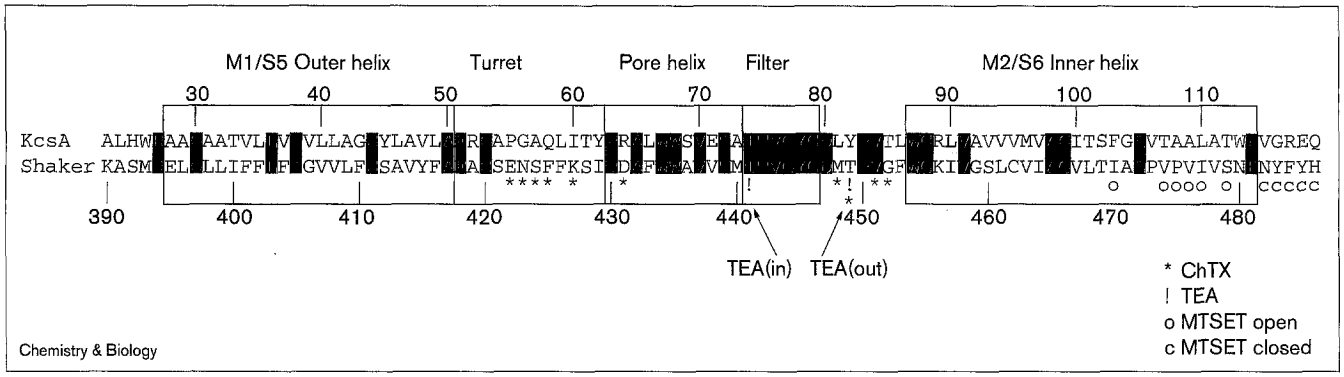


The discovery of naturally occurring venom peptides targeted to K^+ channels provided a precision set of molecular tools to identify various isoforms of K^+ channels and to probe the structure of the external vestibule. Charybdotoxin (ChTX), a 37-residue peptide toxin from the scorpion *Leiurus quinquestriatus* was first described as an external blocker of large conductance Ca^{2+}-activated K^+ channels [15]. It later became clear that ChTX is just one member of a large family of homologous scorpion toxins that bind to many different types of K^+ channel proteins [16]. The binding affinity of a particular ChTX homolog for a particular type of K^+ channel depends on natural substitutions of amino-acid residues at key positions on one face of the toxin and in the outer vestibule of the channel. Several groups used information derived from detailed studies of this toxin–channel interaction to construct a map of the external vestibule (e.g. Figure 2d). The Lys27 residue of ChTX was found to interact with K^+ ions flowing through the pore from the inside [17], positioning Lys27 of the toxin at the very outer entrance of the K^+ selectivity filter. Other pairs of residues on the toxin and the channel were found to interact directly or reside together in close proximity in the bound state using a pairwise mutagenesis technique known as thermodynamic mutant cycle analysis [18]. Other charged residues on the toxin and channel were found to interact electrostatically. The use of ChTX was also important in identifying the particular region within the linear sequence of the K^+ channel protein that forms the outer part of the pore and the selectivity filter [19]. This region, located on an external linker between two adjacent transmembrane spanning elements, is called the pore region or P-region (Figures 3,4). As this segment appeared to comprise a pore-forming loop connecting two adjacent transmembrane α helices, this part of the K^+ channel is also described as a pore loop [20]. Although K^+ channel toxins evolved as electrophysiological poisons to help scorpions immobilize their prey and ward off enemies, they have been invaluable for structure–function analysis. They were used successfully to predict the protein-folding pattern and the relative location of numerous amino-acid residues in the outer mouth of K^+ channels (Figures 4,5).

Molecular biology sends in the clones

The first examples of channel proteins to be cloned with a structural design containing the P-region element were voltage-gated Na^+ channels (Na_V) [21] and Ca^{2+} channels

Figure 4



Sequence alignment of residues 23–119 in the KcsA crystal structure with the S5-P-S6 region of the *Shaker* K_V channel. Sequences and residue numbering correspond to PIR accession numbers S60172 (KcsA) and S00479 (*Shaker*). Identical residues are shaded red and chemically similar residues are yellow. A more extensive alignment of the whole KcsA sequence with other K^+ channel proteins can be found in the original cloning paper of Schrempf *et al.* [48]. Boxes correspond to residues found in the three α-helical regions of KcsA (M1, pore helix, M2), an external loop called the turret, and a K^+-binding region called the selectivity filter. Residues of *Shaker* that interact with charybdotoxin in the outer vestibule, interact with TEA^+, and react with MTSET in closed and open states are marked.

(Ca_V) [22]. Ironically, these channels are the most complex members of the superfamily from a structural standpoint. A key feature of Na_V and Ca_V proteins is an internal repeat of four homologous S1–S6 motifs composed of six transmembrane-spanning segments (Figure 1). The first actual K^+ channel member of the family to be cloned was a voltage-gated K^+ channel (K_V) of *Drosophila* corresponding to the *Shaker* mutant [23]. This protein contains only a single S1–S6 motif. An ingenious mixing experiment using wild-type *Shaker* and a ChTX-insensitive mutant of *Shaker* established that functional K_V channels are formed as a radially symmetric tetramer of four monomers, with the ion channel located at the central subunit interface [24]. This finding correlated nicely with the pseudotetrameric design of the distantly related Na_V and Ca_V channels. As described above, the sensitivity of the cloned *Shaker* channel to toxins of the ChTX family provided an ideal probe and template system for discovery of the P-region by mutational analysis. Using the *Shaker* clone, the P-region was first identified as an external loop between transmembrane segments S5 and S6 [19].

At present, ~20 distinct genes encoding members of the K_V channel family have been described in mammalian species. These K_V genes apparently represent nine distinct subfamilies, K_V1 to K_V9, classified on the basis of homology [25]. All of the cloned P-region channels mentioned so far are activated as a steep function of membrane voltage. These channels all contain a characteristic S4 segment with five to seven arginine or lysine residues at every third position. This positively charged S4 segment functions as a structural component of the voltage-sensing mechanism. Cloning work has also yielded P-region channels with a conserved S1–S6 motif that are activated by intracellular ligands. Examples of these channels are large conductance Ca^{2+}-activated K^+ channels (maxi K_{Ca}) [26] and cyclic nucleotide-gated channels (CNG) [27]. These two proteins have unique cytoplasmic domains located at the carboxy-terminal side of S1–S6 that contain the binding site for activating ligands, Ca^{2+}, and cyclicGMP or cyclicAMP, respectively.

Members of the P-region family having the simplest protein structure were first identified with the cloning of the inward rectifier family of K^+ channels (K_{IR}) [28]. Inward rectifier channels are so named because their current–voltage behavior exhibits more K^+ current in the inward than in the outward direction. These highly K^+-selective channels lack the S1–S4 part of the S1–S6 motif of K_V channels. They consist simply of a segment called M1–P–M2 that is homologous to the S5–P–S6 region of K_V channels. K_{IR} channels lack the finely tuned voltage-sensing mechanism of K_V channels but retain the ion-conduction pathway. At present, at least six distinct subfamilies of cloned K_{IR} channels are recognized [29]. On the simple basis of structural similarity, the prokaryotic KcsA channel is apparently a distant relative of the K_{IR} family. Additional types of K^+ channels have been cloned that appear to be combinations of K_V and K_{IR} channels. For example, the TOK1 clone of yeast is a tandem combination of an S1–S6 motif and a KIR-like motif [30]. Also, the human TWIK-1 K^+ channel clone is a tandem combination of two K_{IR}-like motifs [31]. As TOK1 and TWIK-1 proteins contain two P-region pore domains, it is presumed that both of these functional K^+ channels are formed as dimers. The fact that all of these diverse types of channel proteins utilize a version of the M1–P–M2 domain of a structurally simple channel, such as KcsA, to

form the ion-conduction pathway implies that the pore-forming domain is a protein module (Figure 1; e.g. a structural element that has essentially been spliced together with other protein domains by an exon shuffling process in the course of molecular evolution).

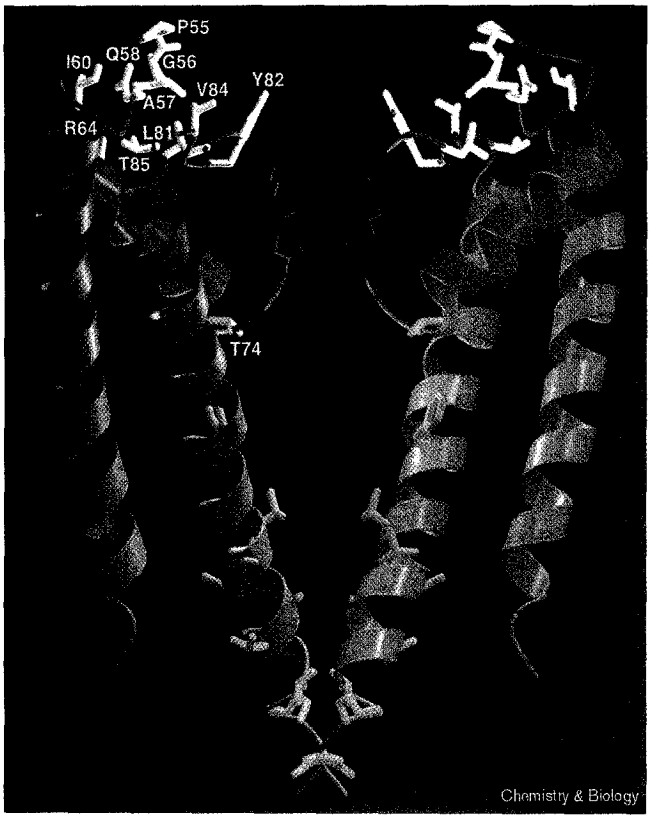
When the P-region sequences of all the above mentioned K^+ channels are compared, a remarkable pattern is found. All K^+-selective channels contain a highly conserved sequence motif within the P-region. In the *Shaker* K_V channel this sequence is TMTTVGYG (using single-letter amino-acid code). Mutational analysis demonstrated that this particular region, called the signature sequence, contains the most crucial residues for K^+ selectivity [32]. CNG channels, which are nonselective cation channels, contain a two-residue deletion of Tyr–Gly (YG) in this signature sequence. This deletion has been shown to be the structural difference responsible for disruption of strict K^+-selectivity [33]. Ca^{2+}-selective Ca_V channels and Na^+-selective Na_V channels have a different type of signature sequence in the P-region. Ca_V channels contain four aligned glutamate residues in the four homologous S1–S6 domains (D1–D4), whereas Na_V channels contain an aligned motif of Asp, Glu, Lys, Ala in D1–D4 [34]. The latter conserved motifs of charged residues have also been shown to specify the essential ionic selectivity properties of these channels. The recognition of a highly conserved signature sequence in the P-region of all known K^+ channels therefore essentially meant that the first solved crystal structure of any P-region protein containing this motif ought to reveal the structural and chemical basis for K^+ selectivity.

Stuck in the wilderness with mutagenizers, SCAM artists and fashionable models

The availability of many interesting K^+ channel clones and specific scorpion toxins with solved nuclear magnetic resonance (NMR) structures, coupled with the *Xenopus* oocyte expression system and powerful electrophysiological recording techniques, resulted in a frenzy of K^+ channel prospecting not unlike the California gold rush. At times, the prodigious yield of publications from the 'K^+ rush era' (1987–1998) seemed to foster the illusion that the exact three-dimensional structure of the *Shaker* protein would be completely solved by brute functional analysis without any real structural data. In these colorful times, ingenious mutational strategies were employed to transform the biophysicists' black and white cartoons into vivid models of folded proteins complete with sidechains. Mutational analyses of the K^+ channel signature sequence [32], the external ChTX binding site [16], and the external and internal blocking sites for TEA^+ [35–37] provided valuable information (Figure 4) that was used to hypothesize how the P-region folded to produce a K^+-selective pore (e.g. Figures 2e,4,6).

In retrospect, one of the most significant findings was the identification of two threonine residues of *Shaker* that

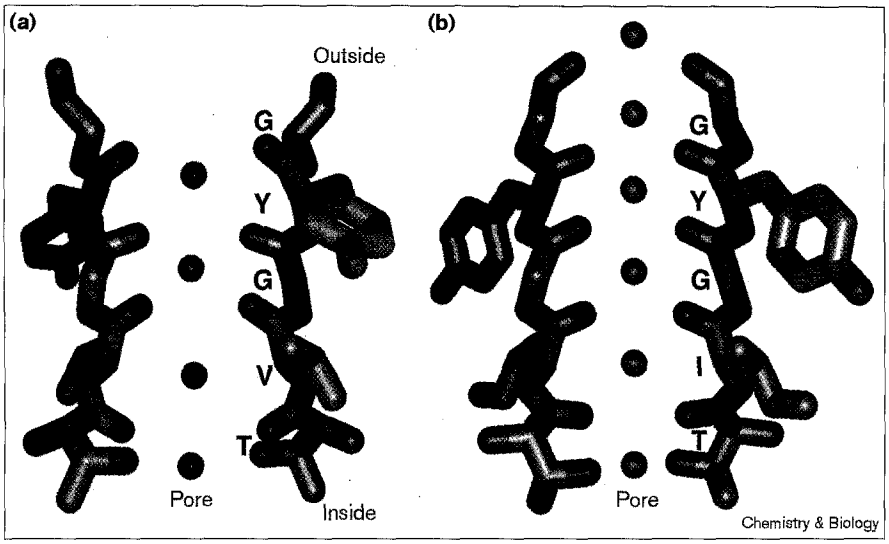
Figure 5



Ribbon diagram of two monomers of the KcsA structure showing the location of various residues functionally mapped in the *Shaker* K_V channel. Residues that interact with toxin homologs of charybdotoxin, white; external TEA^+, yellow; internal TEA^+, mustard; $MTSET^+$ in the open state, pink; $MTSET^+$ in the closed state, green; GYG mainchain of the selectivity filter, red. This figure is reproduced from [1] with permission.

control the blocking affinity of TEA^+. The affinity for external TEA^+ in K_V channels was found to be determined largely by the amino-acid residue at Thr449 (*Shaker* numbering) [35,37]. The wild-type *Shaker* channel, which has Thr449, has an IC_{50} for external TEA^+ block of ~22,000 μM. *Shaker* mutants with tyrosine or phenylalanine residues at this position have lower IC_{50} values for external TEA^+ of 650 μM and 350 μM, respectively. As K_V channel isoforms with naturally occurring tyrosine residues at this position also show high affinity for TEA^+, this site is the likely basis for the natural variation in sensitivity to external TEA^+ of different K^+ channels. The results of tandem dimer experiments suggested that Tyr449 residues on all four subunits contribute equally to the binding energy of external TEA^+ [35]. This finding led to the idea of a cage of four aromatic rings of tyrosine engaged in a cation-π orbital interaction with one sandwiched TEA^+ molecule situated at the outer entrance to the selectivity filter. The second important threonine residue is 441 of *Shaker*. The mutation of Thr441→Ser was found to increase the IC_{50}

Figure 6



Close-up view of the K^+ channel selectivity filter. **(a)** A stick representation of the highly conserved residues TVGYG in the crystal structure of KcsA. Three K^+ ions (blue spheres) and a water molecule (red sphere) are shown as described by Doyle *et al.* [1]. The bottom two K^+ ions are believed to represent the locations of a single K^+ ion in equilibrium between two sites. The narrow region of the filter appears to present four rings of backbone carbonyl oxygen atoms from the TVGY residues that are suitable for coordination of K^+. The hydroxyl oxygen atoms from the bottom four threonine residues are also potential K^+ ligands. **(b)** An earlier hypothetical model of Guy and Durell [49] of the selectivity filter region of the ROMK1 inward rectifier K^+ channel for comparison. This figure was kindly prepared by H. Robert Guy.

value for block by internal TEA^+ from 700 μM in the wild-type channel to 7400 μM in the serine mutant [36]. In the simplest interpretation, these data imply that Thr441 is located at the internal TEA^+ blocking site and Thr449 is at the external TEA^+ site. This information lent real credence to the notion that the amino-terminal half of the P-loop, which emerges on the outside of the membrane from S5, actually dips back into the membrane half-way and then turns to the outside again at Thr441 (internal TEA^+ site), with the signature sequence forming the narrow selectivity filter, before opening up to the external surface at Thr449 (external TEA^+ site). Remarkably, this simple-minded interpretation of such inherently risky mutagenesis experiments turned out to be practically correct when compared to the actual location of the corresponding residues (Thr74 and Tyr82) of the KcsA channel in the crystal structure (Figures 3,5).

Another popular approach that contributed much useful information towards the prediction of K^+ channel structure is known by the dubious-sounding acronym of SCAM, the substituted-cysteine accessibility method [38]. In this technique, a cysteine residue, introduced by mutation at any position of a channel protein, is tested for reactivity using an electrophysiological assay with a charged derivative of methylmethanethiosufonate (e.g. $MTSET^+$) that is highly reactive with a free thiol group of cysteine. In principle, if the substituted cysteine residue lies on the water-accessible surface of the protein, it should react rapidly with $MTSET^+$, whereas a buried residue should be nonreactive or slowly reactive. Such SCAM experiments have been used effectively to develop predictions for the peptide backbone fold of the P-region and the relative degree of aqueous exposure of key residues for the *Shaker* clone.

In a particularly provocative set of studies, most of the residues at the carboxy-terminal half of the S6 membrane span of *Shaker* were tested for accessibility to modification by $MTSET^+$ [39]. The tested residues appear to fall into two classes. Residues 478 and 482–486 react rapidly with $MTSET^+$ at a similar rate, regardless of whether the channel is in the closed or open state. Residues 470 and 474–477 are relatively nonreactive in the closed state but increase their rate of reactivity with MTSET by many orders of magnitude in the open state (Figure 5). These data support the idea that at least part of the S6 transmembrane segment forms the internal lining of the gated pore. Furthermore, such results suggest that *Shaker* residues 470–477 are located in a deep region of the ion-conduction pathway that is accessible to blockers and cations (K^+ and $MTSET^+$) entering from the inside only when a conformational change results in the opening of an internal gate. In contrast, residues 482–486 would appear to reside on an exposed intracellular surface that is outside the gated region of the pore [39]. In viewing the location of the corresponding S6 residues of *Shaker* on the M2 (inner helix) of the KcsA structure (Figure 5), this daring interpretation appears to have immediate relevance for a hypothetical gating mechanism that involves movement of the inner helix bundle.

In the pre-KcsA era, the deluge of information on K^+ channel sequences, mutations and substituted-cysteine reactivity left many workers struggling to make structural sense of it all. In trying to interpret all of this data in a self-consistent manner, several groups adopted a protein-modeling approach. The greatest problem facing model builders was the lack of a known protein fold on which to base the model. One of the most ambitious efforts used known principles of membrane protein structure, sequence

analysis, and mutagenesis data to derive a plausible structure of several K^+ channels, such as the *Shaker* K_V channel and the KcsA channel [40,41].

In the construction of such models, it was assumed that the transmembrane segments would be α helical, as found in known structures of membrane proteins such as bacteriorhodopsin and the bacterial photosynthetic reaction center. The large database of K^+ channel sequences was used to classify residues in the transmembrane segments according to the degree of sequence conservation. Poorly conserved hydrophobic residues found on the same face of a helical wheel representation of the presumed helices were proposed to contact the lipid hydrocarbon [40]. The amino-terminal half of the P-region was also proposed to have an α-helical secondary structure based on the propensity of helix forming residues and mutagenesis data. As structural models for K^+ channels were proposed and increasingly refined, the channel field seemed to be seduced by the rational beauty of the models but unwilling to place much faith in their fidelity. Now that the structure of KcsA has appeared, one can readily compare the real thing with its hypothetical counterpart. A rigorous comparison of the latest detailed model and the KcsA structure deserves far more comment than can be given here, but even a superficial glance shows that the model [40] bears a significant resemblance to the reality (Figures 2e,3,6). One of the major differences in the proposed model of the closed conformation of KcsA is the choice of a kinked α helix for the M2 transmembrane segment. This was proposed because many K^+ channels such as *Shaker* have two proline residues in this region of S6 (Figure 4). The KcsA molecule itself does not have proline residues in M2. Its structure is a regular α helix tilted at an angle of 25° with respect to an axis perpendicular to the plane of the membrane. It remains to be seen whether K^+ channels with proline residues in S6 actually do have distorted M2/S6 helices and whether this helix conformation changes in the open versus the closed state. Overall, the good news for model building is that indirect structural data from all of the investigative techniques that are now available can be interpreted, with appropriate caution, to fashion a hypothetical structure that is fairly close to the real thing.

Things finally crystallize: in the clearing stands an upsidedown teepee

Hardly anyone seriously believed that it would be possible to crystallize a K^+ channel protein. But that did not stop the laboratory group led by Rod MacKinnon at Rockefeller University [1]. They expressed the recombinant KcsA protein in *Escherichia coli*, solubilized and purified it in decylmaltoside detergent, cleaved off ~35 carboxy-terminal residues with chymotrypsin, exchanged the purified truncated protein into another detergent (*N*,*N*-dimethyldodecylamine-*N*-oxide, LDAO) and found conditions for growing crystals suitable for X-ray diffraction. Heavy atom (mercury) derivatives were obtained with cysteine substitution mutants and the crystal structure of KcsA residues 23–129 was solved by crystallographic analysis to a resolution of ~ 3.2 Å (Figures 3,5) [1].

What is it about the structure of KcsA that convinces the most hardened biophysicist skeptic that this is really it, that this is how a much-imagined K^+ channel molecule ought to look? The answer is just about everything. In fact so much work has been done to infer the structure of the K^+ channel pore that Doyle *et al.* [1] were essentially able to provide a guided tour of each part of the molecule and explain its functional significance. As expected, KcsA is a tetramer of four identical subunits that form a pore at the center. The fact that the tetramer in the crystal exhibits perfect fourfold symmetry greatly facilitated crystallographic analysis by allowing fourfold averaging. The protein fold of the tetrameric complex is officially described as an 'inverted teepee architecture'. (This is not a jest.) The structural analogy is based on the similarity of four tilted M2 α helices that form a cone supporting the K^+ channel pore to the poles of a traditional cone-shaped tent dwelling of Native Americans (called a teepee) that cross just below the apex.

The KcsA monomer itself is actually a compact bundle of three α helices: the outer transmembrane helix (M1, residues 28–50) that faces the boundary lipids, the short pore helix (residues 63–73 of the P-region) and the inner transmembrane helix (M2, residues 87–114). The ~30-residue P-region itself has three distinct architectural features. Starting from the carboxy-terminal end of M1, the first ~12 residues of the P-region rise from the membrane to form an extended loop called the turret, the next ~11 residues form the pore helix that is tilted inward towards the center of the bilayer, followed by six residues of the selectivity filter and another short loop that finally connects to the amino-terminal end of M2. If the region of the KcsA sequence comprising M1–P–M2 is aligned with the corresponding S5–P–S6 region of the *Shaker* K^+ channel, one finds only 34% amino-acid identity over 89 residues. A similar alignment of just the P–M2 region of KcsA with P–S6 of *Shaker*, however, exhibits 42% identity (66% similarity) over 53 residues (Figure 4). This high degree of sequence conservation in a thoroughly mutagenized region allows direct comparison of residues in KcsA with those found previously to affect specific functions in *Shaker*. Figure 5 shows the corresponding location of *Shaker* residues that affect external scorpion toxin binding, alkali cation selectivity, external and internal TEA block, and reactivity to internal $MTSET^+$, as mapped onto the KcsA structure. Without belaboring the obvious, all 25 of these important residues are located in an eminently logical place for the corresponding function. This structure–function correspondence establishes that evolutionarily distant

K^+ channel proteins (i.e. prokaryotic compared with eukaryotic) undoubtedly have a very similar protein fold for the pore domain. To further emphasize this point, MacKinnon *et al.* [42] were able to engineer the KcsA molecule competent to bind a scorpion toxin by mutating just three residues in the P-region of KcsA to amino acids that are amenable to toxin binding in the K_V channel family. The binding surface of a scorpion toxin molecule can also be readily docked to the outer vestibule of the KcsA structure with appropriate matching of pairs of residues of the toxin and channel that have been found previously to strongly interact using the method of thermodynamic mutant cycle analysis [18,42]. In summary, K^+ channel followers have every reason to feel elated and relieved that the mutagenic onslaught and SCAMing of their venerated protein was not perpetrated in vain. The functional analyses correlate rather beautifully with the KcsA structure.

What about the ions? Are their energetic requirements satisfied in this story? This is perhaps the most exciting and revealing part of the show. Although the resolution is not quite sufficient to orient carbonyl oxygen bonds, it is all but certain that the 12 Å long selectivity filter at the outer third of the channel is lined by a series of four rings of four mainchain oxygen atoms (Figure 6). On the basis of data from difference density maps using crystals exchanged with the electron dense alkali cations Rb^+ and Cs^+, the selectivity filter of the KcsA channel was found to contain two naked and bound K^+ ions located 7.5 Å apart [1]. The diameter of this narrowest part of the pore is, apparently, just perfect for the coordination of dehydrated K^+ ions by the adjacent rings of carbonyl oxygen atoms. If the filter is rigid, smaller Na^+ and Li^+ ions would not be able to achieve stable coordination with the carbonyl oxygen atoms and thus would not compete effectively with K^+ for binding to the selectivity filter. Amazingly, just beyond the narrow filter towards the intracellular side, there is a wide cavity in the center of the channel that is ~10 Å in diameter and large enough to contain about 60–100 H_2O molecules. There is also density consistent with a third K^+ ion at the center of this water-filled cavity, giving a total of three K^+ ions found in the crystal structure of this now confirmed multi-ion pore. The large central cavity and the 18 Å long inner tunnel leading to it is primarily lined with hydrophobic chemical groups. This design of the inner pore makes it easy to imagine the mechanism underlying penetration and block of the channel from the inside by TEA^+ and its larger derivatives such as $TPeA^+$ and C_{10}-TEA^+.

The original paper [1] discusses at greater length why the structure of the ~45 Å long pore of KcsA comprises an efficient device for selectively binding multiple K^+ ions and catalyzing their conduction through the membrane. Stabilization of K^+ in an electrostatically customized protein passageway across the low dielectric membrane barrier is achieved by a combination of the following features: direct coordination with the electronegative carbonyl oxygen atoms of the selectivity filter, additional charge stabilization provided by interaction of K^+ with the negative dipoles of the four pore helices oriented towards the center of the channel, polarizable solvent molecules in the large water-filled central cavity and the aqueous internal tunnel leading into it. The whole process of molecular sieving and selective binding of K^+ is mediated only by the short 12 Å filter region that comprises just a third of the membrane thickness. Aside from the selectivity filter and the attractive electrostatic influence of fixed negatively charged residues at the entrance to the inner and outer vestibules, the rest of the channel does not appear to provide any sites for specific chemical interactions with inorganic cations. It merely serves to enhance the diffusion of ions in a hydrated form. Another unexpected feature of the structure worth mentioning and investigating is a network of 12 aromatic sidechains (eight tryptophan and four tyrosine residues) that encircle the selectivity filter. These residues are proposed to serve as a kind of bondage device that constrains the filter to a proper diameter for K^+-selective coordination [1].

Getting back to the future

After the thrilling climax reached with the first crystal structure of a K^+ channel, what other conquests can possibly arouse the intellectual passions of chemical biologists interested in ion permeation across membranes? As the ion-conduction pathway is essentially solved, the determination of the structure of the complete S1–S6 domain of a K_V channel is a worthy objective because this will reveal the protein fold of the voltage-sensing element(s) and suggest how this part of the protein controls the gating process. The K^+ channel field is already anticipating that a K_V protein structure will contain a helical, charged S4 segment that physically moves through its own 'gating pore' in response to membrane voltage. The related Ca^{2+}- and Na^+-selective voltage-gated channels are also a big item on the long-term, technically formidable agenda, because the P-regions of these channels use a different type of mechanism for tuning their particular versions of ionic selectivity based on a set of conserved charged residues in the filter region.

The K^+ channel pore itself holds still more secrets. Recent work has shown that certain types of K^+ channels lose their strict K^+ selectivity more readily than others when exposed to K^+-free medium [43–46], suggesting that K^+ itself is a structural requirement for its own selectivity. In the absence of K^+, the structure of the K^+ selectivity filter apparently changes, perhaps loosening to permit other cations such as Na^+ to pass through. With an actual structure of a K^+ channel to guide experimentation in answering such questions, the testing of specific hypotheses by site-directed mutagenesis can be carried out more rationally and analyzed with deeper physical insight than ever before.

Now that the field of P-region channels has left the shrouded world of the biophysicists' black box and entered the realm of real protein structure, one can expect that answers to many structural questions in channel biophysics will eventually be visualized at the molecular level and not simply modeled or imagined. In reflecting on the ultimate significance of the KcsA structure in the unfolding drama of ion-channel research, there is no better way to summarize this noble accomplishment than to badly misquote the dark prince, Hamlet, voice of a sage playwright, "The pore's [sic] the thing".

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