

Structure and Anticipatory Movements of the S6 Gate in K_v Channels

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Potassium channels are a diverse family of membrane proteins that in humans are encoded by 75 distinct genes. The ion conduction pores in these channels are well conserved, supporting remarkable ion throughput rates (10^6 – 10^7 s⁻¹) in the face of exquisite selectivity for their namesake ion. Although some potassium channels are always open, more typically the ion conduction pore changes conformation between open and closed states in response to a biological stimulus. In the voltage-activated (K_v) variety of potassium channels, the opening and closing of the channel is driven by movements in the voltage sensing domains, a topic that continues to sustain an energized discussion (Jiang et al., 2003; Ahern and Horn, 2004; Swartz, 2004; Bezanilla, 2005; Long et al., 2005b). Although the structure and movements of the gate region in K_v channels also has been the subject of debate (del Camino et al., 2000; Webster et al., 2004), the new X-ray structure of the Kv1.2 channel (Long et al., 2005a) has helped to clarify several key points. In the current issue of the Journal, del Camino and colleagues (see del Camino et al. on p. 419 of this issue) extend our understanding of the intracellular gate in Shaker by addressing whether it moves in response to voltage sensor activation in anticipation of pore opening. Although previous studies point to the existence of an activated-not-open (or preopen) state in K_v channels, del Camino et al. are the first to provide evidence that the intracellular gate moves before the channel opens.

Over the past few decades strong evidence has been amassed for the presence of an activation gate at the intracellular end of the pore in K_v channels. Studies on the block of K_v channels by intracellular quaternary ammonium (QA) compounds show that a gate can prevent blocker access to the pore in the closed state and that a gate could be closed behind the blocker, effectively trapping it within the channel (Armstrong, 1966, 1969, 1974; Holmgren et al., 1997). Yellen and colleagues went on to explore the state dependence for reaction between water soluble methanethiosulfonate (MTS) reagents applied to the intracellular side of the membrane and Cys residues introduced throughout

the COOH-terminal end of S6 (Fig. 1), uncovering a pattern consistent with this region forming the intracellular gate (Liu et al., 1997). Reactions between Cd²⁺ or Ag⁺ and introduced Cys residues also exhibit exquisite state dependence (del Camino and Yellen, 2001), strengthening the case for the gating of ion movement by the intracellular end of S6. Although reactions between MTS or Cd²⁺ ions and introduced Cys residues typically result in inhibition of the channel, a very different phenotype is observed for V476C (Fig. 1). When Cd²⁺ is added to this mutant channel, it locks the channel in the open state by forming an intersubunit metal bridge between V476C and H486 (Holmgren et al., 1998; Liu et al., 1997), providing a crucial structural constraint for the open state of the Shaker K_v channel. The X-ray structure of the KcsA potassium channel (Doyle et al., 1998) supports the notion of a gate at the intracellular end of the pore because the inner helices in KcsA (equivalent to S6 in K_v channels) form a closed bundle at the intracellular end of the pore. Closer examination, however, reveals that there are several reasons to think that the intracellular gate in KcsA and Shaker may be different. First, Shaker contains a PVP motif that is well conserved in many eukaryotic K_v channels, yet absent from KcsA (Fig. 1). Pro residues often bend or kink α -helices (MacArthur and Thornton, 1991), so a structural variation in the gate region between KcsA and Shaker K_v would not be surprising. Second, it is not possible to accommodate the V476C-Cd²⁺-H486 bridge if the structure of the intracellular region of Shaker looks like it does in KcsA (del Camino et al., 2000). Granted the KcsA is closed and the bridge constraint in Shaker is for the open state, but the situation doesn't improve when the structure of the open MthK channel is considered (Jiang et al., 2002a). The distance between the β carbons of the 476 and 486 counterparts are ~ 15 Å apart in KcsA and >20 Å apart in MthK, too distant for a high affinity bridge involving Cd²⁺ (see below). Third, QAs can protect introduced Cys residues from reacting with MTS reagents, and the pattern of this protection points to an enlargement of

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Abbreviations used in this paper: 4-AP, 4-aminopyridine; MTS, methanethiosulfonate; QA, quaternary ammonium.

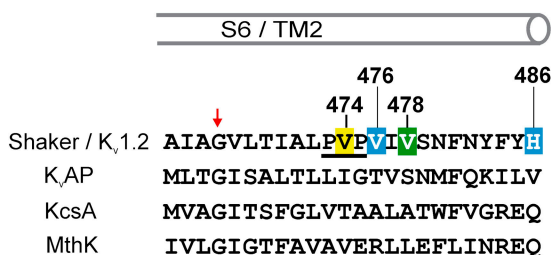


Figure 1. Sequence alignment of the S6 gate region in potassium channels. Numbering for the four highlighted residues is for Shaker.

the pore below the PVP motif (del Camino et al., 2000), an inference that is born out by experiments with bulky MTS reagents (del Camino and Yellen, 2001). To explain these apparent inconsistencies between the structure of KcsA and the biophysical experiments on Shaker, Yellen and colleagues proposed that the PVP induces a bend in the S6 helix (del Camino et al., 2000).

Another conundrum concerns the region of S6 that serves as a hinge or pivot for motions of the intracellular gate. MacKinnon and colleagues (Jiang et al., 2002b) proposed that the structure of the intracellular gate moves from a KcsA-like structure, when closed, to an MthK-like structure, when open, involving a relatively large splaying motion of the inner helices about a well-conserved Gly residue proposed to serve as a hinge (Fig. 1, red arrow). Although this Gly is a particularly sensitive region of the S6 helix in the Shaker K_v channel (Ding et al., 2005), movement about the Gly hinge is incompatible with other evidence that V474 within the PVP motif (Fig. 1) does not move much during channel opening. Yellen and colleagues (Liu et al., 1997) showed that Cd²⁺ can be simultaneously coordinated by Cys introduced at 474 in at least three of the four subunits (here designated the 474C(≥3)-Cd²⁺ bridge). When the voltage dependence of Cd²⁺ release from V474C is examined using dimercaptopropane-sulfonate (DMPS), the rate of release tightly follows the voltage dependence of channel opening (Liu et al., 1997; Webster et al., 2004), suggesting that a gate below 474 regulates the access of DMPS. However, the absence of an effect of Cd²⁺ (bound at 474) on the open-closed equilibrium suggests that this position within the PVP motif does not move much during gating and that the diameter of the intracellular pore must be much smaller than the 20 Å seen in the open MthK channel.

The recent X-ray structure of the Kv1.2 channel (Long et al., 2005a) represents a remarkable step forward because this mammalian homologue of Shaker contains the much discussed PVP motif. There are many noteworthy features of the new structure, but

three are most relevant for the present discussion: (1) although it seems clear that the Kv1.2 channel is in an open state, the S6 helices are not nearly as open as they are in either KvAP or MthK (Long et al., 2005a); (2) although the S6 segment remains helical at the PVP motif, it is a relaxed helix, resembling a spring that has been stretched out a portion of its length, and it is indeed bent (Fig. 2); and (3) the structure of the S6 region in Kv_{1.2} is compatible with the distance constraints from both the 476C-Cd²⁺-H486 and 474C(≥3)-Cd²⁺bridges in Shaker (Fig. 2). The distance between C^β atoms of V476 and H486 are 9.3 Å apart. (The side chain densities are generally not well defined in the intracellular region of S6 so the following measurements were made between C^β atoms without considering side chain geometry.) Taking into account a C^β-S distance of 1.8 Å and a C^β-N^ε distance of 3.7 Å, leaves 3.8 Å between the S^γ of 476C and the N^ε of H486, clearly within range for coordinating a Cd²⁺ ion whose ionic diameter is 1.94 Å. The distance from the C^β atoms of V474 to the central axis of the pore is 5.9 Å. In this instance, taking into account C^β-S distances of 1.8 Å and the Cd²⁺ ionic radius of 0.97 Å, leaves 3.1 Å between the S^γ of 474C and a Cd²⁺ at the central axis of the pore, within a fraction of an angstrom of what is expected for S-Cd²⁺ bonds. (Bond lengths for Cd²⁺ coordination by S are around 2.5 Å [Rulisek and Vondrasek, 1998; Enescu et al., 2003].) This agreement between the distance constraints obtained from careful biophysical experiments and the X-ray structure of Kv1.2 is really quite remarkable, establishing the bent S6 model and the smaller open pore dimensions for K_v channels in the Shaker family.

The latest chapter on the activation gate from Yellen and colleagues (del Camino et al., 2005) focuses on whether the S6 gate moves after voltage sensor activation, but before it actually opens to support ion conduction. Extensive characterization of gating currents, and both unitary and macroscopic ionic currents, suggest that K_v channels traverse many distinct states between negative membrane voltages, where the voltage sensors are resting and the gate is closed, and depolarized voltages, where the voltage sensors are activated and the gate opens (Bezanilla et al., 1994; Hoshi et al., 1994; Stefani et al., 1994; Zagotta et al., 1994a,b; Schoppa and Sigworth, 1998a,b,c). In the course of investigating how the gating mechanisms of the *Drosophila* Shaw and Shaker K_v channels differ, Aldrich and coworkers described a triple mutant of Shaker (named ILT) where three positions in S4 were mutated to the corresponding residues in Shaw (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). In ILT, the voltage ranges over which the voltage sensor movement and the channel opening occur are quite well separated; the bulk (~87%) of gating charge movement oc-

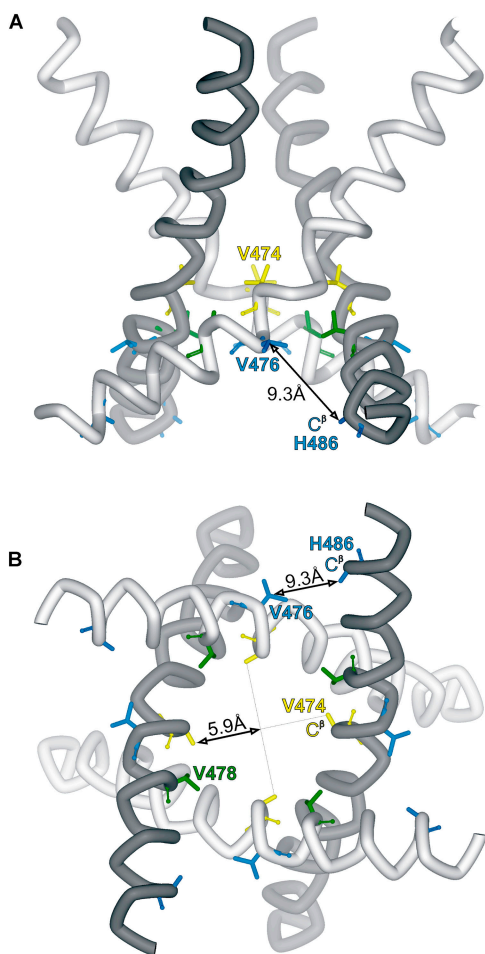


Figure 2. S6 helices from the X-ray structure of Kv1.2. (A) Side view of the S6 helices. Cd²⁺ bridges 476C and H486 (blue side chain atoms) between adjacent subunits, locking the channel open. Only one of four possible bridges is shown and only the β-carbon of H486 is shown in the model. Distance between C^β atoms of V476 and H486 is 9.3 Å. (B) Intracellular view of the S6 helices. A second type of bridge is shown where V474C residues coordinate Cd²⁺ at the central axis of the pore. Distance between C^β atoms of V474 across the pore is 11.8 Å.

occurs well negative of 0 mV, and appreciable channel opening occurs only above 0 mV. A small component of the gating charge moves in the voltage range of channel opening, accounting for ~13% of the total charge movement overall. Importantly, if the membrane is depolarized from a holding voltage of 0 mV (where the voltage sensors are already activated), channel opening proceeds along a single exponential time course, as if the channel is primarily traversing a single transition between a final closed state and the open state. A particularly beautiful demonstration of the separation of the main charge moving steps and channel opening was recently reported by Isacoff and colleagues when they attached a fluorophore to S4 and looked at gating charge, fluorescence, and channel opening in the ILT mutant of Shaker (Pathak et al., 2005). Two readily dis-

tinguishable components of fluorescence change are observed for S4, one occurring at voltages negative of 0 mV and correlating with the main component of gating charge movement, and the other at voltages positive to 0 mV and correlating with channel opening.

The paper by del Camino, Kanevsky, and Yellen sets out to use the ILT mutant and 4-aminopyridine (4-AP) as tools to isolate channels in the final closed state before channel opening, which they refer to as the activated-not-open state. They begin by shoring up the case for using 4-AP to isolate the activated-not-open state. Loboda and Armstrong proposed that 4-AP binds within the inner cavity of the pore after the activation gate opens, much like QAs, but in this case the blocker stabilizes the closed state, in effect stabilizing the activated-not-open state of the channel (Kirsch and Drewe, 1993; Kirsch et al., 1993; Armstrong and Loboda, 2001; Loboda and Armstrong, 2001). This model for 4-AP has support from the fluorescence measurements discussed already (Pathak et al., 2005), which show that 4-AP inhibits the component of S4 fluorescence change associated with the opening step in the ILT mutant. The first experiments by del Camino and coworkers formally show that 4-AP requires an open channel to produce block and that it can be trapped in the channel with subsequent hyperpolarization of the membrane. They also show that a QA and 4-AP act competitively, consistent with 4-AP binding in the cavity. This last experiment is particularly elegant because they avoid the confounding effect of 4-AP stabilizing the closed channel (which by itself would make the blockers appear to compete) by looking at competition on channels that have first been locked open using the 476C-Cd²⁺-H486 bridge. Interestingly, they also see that the affinity of 4-AP for locked open channels is far weaker than otherwise observed, consistent with 4-AP stabilizing the activated-not-open state.

With the ILT mutant and 4-AP in hand, del Camino et al. set out to look for movements in the gate region of the channel through an examination of the reaction between MTS reagents and V478C (Figs. 1 and 2). This residue lies within the region of the pore that closes off to prevent ion conduction, yet very close to where the pore widens into the cytoplasm (Liu et al., 1997; del Camino and Yellen, 2001; Hackos et al., 2002; Kitaguchi et al., 2004). For these experiments they use the LT background, a variant of ILT whose opening is not quite as drastically shifted to positive voltages. In LT the reaction rate between MTS reagents and 478C is ~10 times faster in the open state measured at +110 mV when compared with the closed state measured at -120 mV, similar to what has been observed without the LT mutation in S4 (Liu et al., 1997; del Camino and Yellen, 2001). To determine the reaction rate in the activated-not-open state they measure the reaction rate at

−10 mV, where the voltage sensors are activated but the channel is closed, and at +110 mV in the presence of 4-AP. Not only is the reaction rate for the activated-not-open state larger than for the resting/closed state, it is actually over twofold higher than for the open state. In other words, when the voltage sensors activate, the accessibility of V478C increases more than what is observed when the gate actually opens. This result shows that the accessibility of the S6 gate increases with voltage sensor activation, as if the intracellular region of the channel has undergone a conformational change in anticipation of channel opening.

The final experiment provided by del Camino et al. addresses whether the intracellular gate itself remains closed to ions when the channel is in the activated-not-open state. It has been postulated that K_v channels might contain two gates that open in response to voltage sensor movement, the intracellular S6 gate discussed here and another gate of sorts in the selectivity filter of the channel (see Chapman and VanDongen, 2005, and references therein). One possibility is that the main charge-translocating movements of S4 open the S6 gate entirely such that it no longer serves as a barrier to ion permeation, and that when further depolarization causes an additional movement of S4, a different gate opens and allows the channel to conduct ions. Although recent experiments have shown that the activated-not-open state of the gate remains shut to DPMS (Webster et al., 2004) and the inactivation ball (Pathak et al., 2005), these molecules are quite a bit bigger than K⁺ ions. To examine whether the S6 gate remains shut to ions, del Camino and colleagues test whether Cd²⁺ could access V474C (Fig. 1,2), a residue that is positioned just above the bundle crossing in KcsA and whose reaction with Cd²⁺ is strongly favored by channel opening (Liu et al., 1997; del Camino and Yellen, 2001). What they observe is that, in spite of the increased accessibility of V478C in the activated-not-open state, the intracellular gate remains competent to limit the accessibility of Cd²⁺ to V474C. Although the intracellular gate seems to move with voltage sensor activation, it remains shut to the flow of ions. It is as if the large charge-translocating movements of S4 do indeed move the gate, but more along the lines of “awakening” the gate rather than actually opening it. The subsequent cooperative opening step also involves a movement of S4, but this motion results in the translocation of only a small fraction of the total gating charge, as nicely demonstrated by the collective experiments on the ILT mutant of Shaker.

As satisfying as the current picture of gate motions may be, many questions remain. Although the V474(≥3)-Cd²⁺ bridge suggests that this position within the PVP motif remains relatively stationary, it will be interesting to further explore whether the PVP motif acts as the

sole hinge or pivot for movements of the lower S6, or if the gating Gly may also be involved. Some models of gating envision S4 tugging the S6 gate open via the connecting S4–S5 linker, a region clearly important in coupling voltage sensors and the gate (Lu et al., 2002; Long et al., 2005b), yet it is unclear how all four S4s can move the bulk of their gating charge, yet fail to actually open the S6 gate. Although there is a wealth of information about voltage sensor function (Bezanilla, 2005), it will be particularly fascinating to explore how the physical S4 movements relate to the major and minor components of gating charge movement now that it seems clear that both impact the state of the S6 gate, but only the latter actually opens it. The new information on the activated-not-open state will also be useful because it clarifies several strategies for crystallizing the Kv1.2 channel in this state, either using the ILT mutant or 4-AP to stabilize the activated-not-open state. There is much to learn about how the voltage sensors move and how these movements are translated into the opening of the S6 gate, but there is reason to be optimistic that new structures, together with the type of elegant biophysical experiments discussed here, should make a powerful duo in deciphering how it all works.

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