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Answers and Questions from the KvAP Structures

Minireview

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The recent landmark structures of KvAP, a voltage-gated potassium (Kv) channel, provide the first high-resolution experimental structural models of this class of proteins. Previous extensive studies of Kv channels provide a means to evaluate and interpret the KvAP structures. In this minireview, we survey different experimental approaches to Kv channels and map these findings to KvAP, showing that the relationship between the KvAP structures and other Kv channels is uncertain.

Few families of proteins have been studied as relentlessly as the voltage-gated potassium (Kv) channels. Since the cloning of the prototypical family member, Shaker, these proteins have been probed exhaustively by a host of techniques, including electrophysiological recording, scanning mutagenesis, site-specific accessibility studies, crosslinking, and novel fluorometric techniques. All of this work has been aimed at understanding the structures of the channels and how exactly they are able to generate electrical signals by opening and closing in response to changes in membrane potential.

An immense frustration has been that these studies have all been carried out blind, without any detailed structural models of the channels to guide experiments or make sense of results. But that may have all just changed: in a seminal series of papers, the MacKinnon group has reported the cloning, characterization, and high-resolution structure of KvAP, an archaebacterial potassium channel with a sequence and properties similar to its more familiar eukaryotic counterparts (Jiang et al., 2003a, 2003b; Ruta et al., 2003). These papers describe two distinct structures of the channel, which, along with an original accessibility study, form the basis for models of the channel in its open and closed states.

These structures have come about despite two fundamental obstacles in channel crystallography. The first is the considerable difficulty in expressing and crystallizing integral membrane proteins. These problems have been partly overcome by the cloning of channels from bacteria and the subsequent sequencing of full bacterial genomes (including *Aeropyrum pernix*, the thermophile that produces KvAP), advances which have permitted the purification of milligram quantities of membrane proteins. Equally important are the skills of the few groups able to coax crystals out of these proteins, including the MacKinnon laboratory, which continues its spectacular march through the channelome with the KvAP structures.

The second fundamental problem may be more difficult: how do we relate these structures to the nervous system proteins that have been so well studied over the last 50 years? There are two distinct issues here; the first is whether the bacterial protein is like other Kv channels. The happy news is that KvAP is a bona fide voltage-gated potassium channel with high sequence similarity to other Kv channels (Ruta et al., 2003), though there are potentially significant differences to be explored. A thornier problem is in trying to understand what sort of physiological state the KvAP structures represent. This is usually a nonissue for the soluble proteins that have traditionally been fodder for crystallographers: receptors can be crystallized with or without ligand; enzymes with product, substrate, or transition-state analog. But even the simplest channels can have multiple open, closed, and inactive states, each with a possible structural counterpart, and some models require hundreds of states to describe channel properties. An added complication is that channel states may change in response to minor changes in the surrounding lipid and solution composition, let alone to the detergents, salts, and other things necessary for crystals to grow.

Fortunately, decades of studies of Kv channels provide a trove of data against which we may evaluate the KvAP structures. In this minireview, we survey different experimental approaches used to study Kv channels and map these findings to KvAP. Because the potassium channel pore has been discussed at length for the KcsA, MthK, and KirBac1.1 structures (Doyle et al., 1998; Jiang et al., 2002; Kuo et al., 2003), we concentrate here on the novel voltage-sensing domain of the channel.

The KvAP Structures

The KvAP study produced two structures: one is a 3.2 Å structure that consists of the entire membrane region (called "full KvAP" here), and a second is a higher-resolution (1.9 Å) structure of just the voltage-sensing S1 through S4 segments (called "S1–S4" here). Both proteins are complexed with Fab antibody fragments bound to the short loop between S3 and S4, which were added to stabilize the exceptionally dynamic S4 segment for crystallization.

The KvAP structures are surprising in many ways. Previous predictions of the channel membrane-spanning regions were influenced by early membrane protein structures (see http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html and http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html) and were often drawn as six neatly packed transmembrane helices normal to the plane of the membrane. In the KvAP structures, the segments of the voltage sensor are all helical, but none are normal to the membrane, and none even completely transverse the membrane. Some are divided into smaller segments. The full KvAP structure has the S1 and S2 helices both start and end in the interior of the membrane, something we think has not been seen in other structures. (It is important to point out that there

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are no membranes in the KvAP crystals and, probably, no bilayers of any sort but, rather, an uncharacterized dispersion of detergent and bacterial lipid [Caffrey, 2003]. The "membrane" is assumed and its placement determined by the pore-forming region of the channel.) Most surprising is S4, which in the full KvAP structure is divided into two segments, one of which rests just below the membrane-cytoplasm interface, parallel to the membrane plane. This segment forms a helical hairpin with S3b—the authors call this hairpin the *voltage paddle*—and lies at the periphery of the channel surrounded by lipid and solution. The papers note that this is a significant departure from the traditional models of voltage-gated channels, which had surrounded S4 with protein. To be fair, many recent models addressed the possibility that S4 was not entirely surrounded by protein, and some had indeed placed it on the periphery in contact with lipid (Durell et al., 1998; Elinder et al., 2001).

Another unexpected feature is that the full KvAP structure appears to be in a mixed open/closed conformation. Much electrophysiological and accessibility data have shown that depolarization of the membrane causes the arginine-rich S4 segment to move outward, leading to an observable "gating charge" and to opening of the channel pore. The full KvAP structure shows the pore in a partially open state, somewhere between the open MthK conformation and the closed (or partially closed; see Kuo et al., 2003) KcsA conformation. However, S4 is not in the corresponding depolarized state; it lays low with respect to the central pore in what appears to be a hyperpolarized position.

The complicating factors, the authors propose, are the Fab fragments, which are bound to the voltage paddle and determine the ordering of the proteins in the crystal. The papers suggest that packing forces distort the structure of the channel. This is an unusual argument, as crystal packing forces are typically thought to be highly localized, although they can cause some proteins with flexible hinges to lock into particular conformations. But KvAP is exceptional in that it is being oriented in two directions—in one way by the Fabs and another by the channel tetramerization domains in the pore—so that the distortions may be more significant. The obvious question that arises here is just how far these distortions are propagated through the structure.

The S1–S4 fragment provides one means to address this concern, as it is free of the channel tetramerization domain forces. S1–S4 (Figure 1A) and the analogous region from the full KvAP (Figure 1B) show similarly helical secondary structure, with identical voltage paddles. But the structures differ significantly in contacts between helices, a point that becomes even more apparent when mapping mutagenesis data to the structures, as discussed below. S1–S4 shows S4 as a continuous helix that forms salt bridges with S2, something that has also been described in Shaker (Silverman et al., 2003). However, the S1–S4 structure pairs S2 with the C-terminal half of S4, while the Shaker data suggest S2 forms salt bridges with the N-terminal portion of S4, two and four helical turns away. In the full KvAP structure, S2 has no interactions with S4, and it instead contacts the pore domain.

This may stem from a second difference between the full KvAP and S1–S4 structures: the latter crystals were grown near physiological pH, whereas full KvAP crystals were grown under significantly more acidic conditions (pH 5.0 acetate buffer in the crystallization solution). Protonation of acidic S2 residues might disrupt the S2–S4 salt bridge network and, although the pKa values of Asp and Glu side chains are close to 3 in solution, they can be much higher in the membrane. Proton-dependent structural changes in this pH range are well known, seen for example in hemeagglutinin, where acidification disrupts buried salt bridges and leads to helix swapping (Bullough et al., 1994). There are no pH data as yet on the electrophysiology or structure of KvAP that might address this issue.

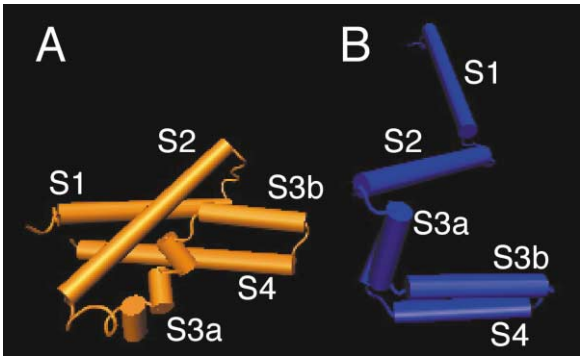

Figure 1. Differences in Tertiary Structure between the KvAP Voltage Sensors Structures

(A) The S1–S4 fragment and (B) the same region from one subunit of the full KvAP structure. The structures have been oriented so that the S4 segments are roughly parallel. VMD has been used for all molecular drawings (http://www.ks.uiuc.edu/Research/vmd/). Note that VMD does not recognize S3a as one continuous helix.

To examine the relationship between the two crystal structures, we mapped data onto them from two Kv scanning mutagenesis studies, which operate under the assumption that positions intolerant of mutation are involved in protein-protein interactions (Bowie et al., 1990) and have been shown to be good predictors of lipid- and protein-facing residues (Fleming and Engelman, 2001). One study used an alanine scan to assess mutation-induced perturbations in voltage dependence for Kv2.1 (Li-Smerin et al., 2000), and a second study probed Shaker using tryptophan substitution in the S1 and S3 segments (Hong and Miller, 2000). In Figure 2, high-impact positions from the alanine scan are indicated in orange, the tryptophan scan in yellow, and residues identified by both studies are shown in red. For S1, all but one position from both scans map to a single face, which in the full KvAP structure points away from the protein-protein interface formed with the central pore (Figure 2A). In contrast, in the S1–S4 fragment this non-tolerant face packs against the conserved S4 helix (Figure 2C). For S3, the red residues localize to the S3a–S3b linker in the full KvAP (Figure 2B), and the least tolerant positions from the alanine study largely cluster on the far side of S4, a region devoid of protein-protein interactions. Much more satisfying is the proximity that the S3 red residues have to the S4 helix in the S1–S4 structure (Figure 2D). These studies suggest that the solo S1–S4 structure provides a better framework to understand tertiary interactions in Kv voltage sensors.

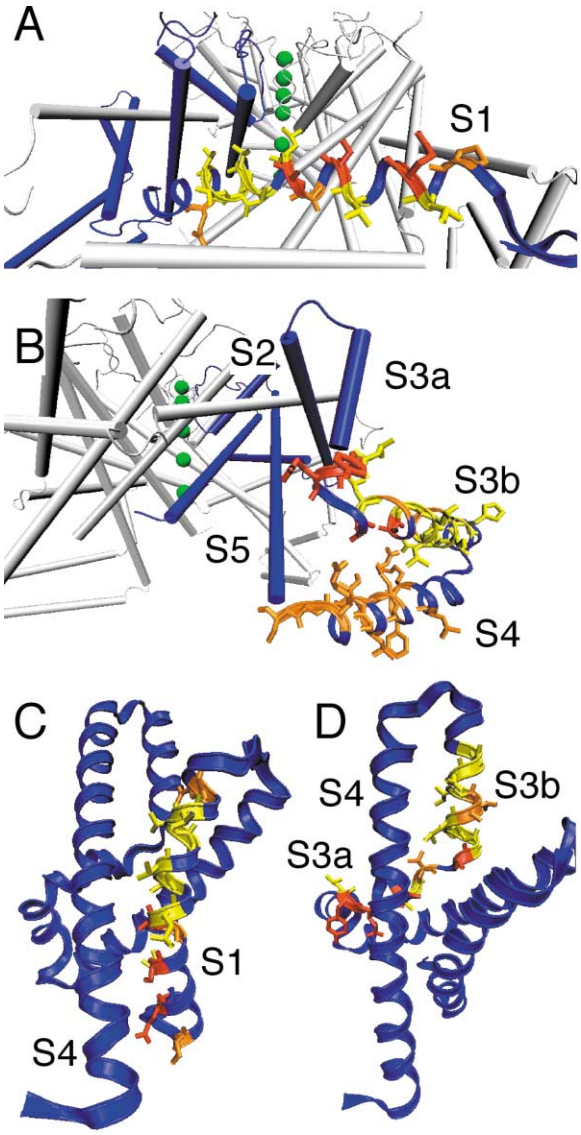

Figure 2. Mutation-Intolerant Residues from Scanning Mutagenesis Studies of the Shaker and Kv2.1 Channels Mapped onto the Full KvAP and S1–S4 Structures

High-impact sites from alanine scans (Li-Smerin et al., 2000) are shown in orange, while the tryptophan scans (Hong and Miller, 2000) are in yellow. Residues identified in both studies are red. Subunits with highlighted residues are in blue, while the remaining subunits are white. (A) Critical residues in S1 projected onto the full KvAP structure. Potassium ions in the central pore are green. Thirteen of the fourteen critical residues face away from the protein. (B) Critical residues in S3 and S4 projected onto the full KvAP structure. High-impact residues in red localize to the S3a–S3b hinge but have no interaction with other portions of the protein. (C) Critical residues in S1 projected onto the solo S1–S4 fragment. (D) Critical residues in S3 projected onto the solo S1–S4 fragment. In both (C) and (D), red residues identified in both studies face conserved regions of S4.

Comparisons with Eukaryotic Kv Channels

The most obvious differences between the KvAP and eukaryotic Kv sequences are in the S1–S2 and S3–S4 loops. Both are short in KvAP and reside deep in the membrane, with the S3–S4 loop being an integral part of the voltage paddle. These linkers are longer in other Kv channels (e.g., Shaker S3–S4 is 22 residues longer), though they vary considerably in length and sequence. Several key pieces of data place them squarely on the extracellular side of the membrane (e.g., Shih and Goldin, 1997). It is unclear how the sequences might form independent domains that snake out and back into the membrane or otherwise fit into the KvAP structure. Other potential differences are examined below.

Is the Voltage Paddle Conserved?

One of the key conclusions of the KvAP study is that the voltage paddle is responsible for carrying the gating charges in response to membrane depolarization. Multiple studies have shown that outward movements of the basic residues in S4 are responsible for the 12 to 13 measured charges that cross the membrane following depolarization (e.g., Seoh et al., 1996). MacKinnon and coworkers propose that S3b and the N-terminal half of S4 cotranslate through the membrane as an unaltered unit during voltage activation. They present a sequence analysis with the conclusion that this motif is highly conserved throughout all Kv channels and, consequently, that the paddle structure and motion are also conserved. However, this contradicts a previous study that compared a series of evolutionarily diverse potassium channels and found S3b to be among the least conserved regions (Durell et al., 1998).

A closer inspection of the alignment in Figure 6b in Jiang et al. (2003a) confirms the previous study, as shown here in Figure 3A. Closely packed residues (within 4 Å) of the S1–S4 structure are connected with solid lines, and side chains found at each position of the 18 channels in the alignment of Figure 6b are shown. No conservation is apparent: all of these sites in S3b are tolerant of polar, nonpolar, and charged residues (except for I106, which has no charged residues). It is also possible that coevolution between residues in S4 and S3b can account for the variability in S3b. However, KvAP S4 positions V119, L122, and R123 have significant interaction with most of S3b, yet the side chain chemistry of these positions is strictly conserved. We also examined an alignment of 84 Kv1 channels from S3b through the S4–S5 linker (as the paper renames the C-terminal half of S4). This is a more direct test of how well the voltage paddle is conserved in eukaryotic channels and avoids problems associated with aligning distantly related channels. As seen in Figure 3B, most of S4 is fully conserved, including hydrophobic positions, as would be expected for a critical region of such closely related proteins. In contrast, S3b is not well conserved, certainly less so than the S4–S5 linker, which the KvAP study characterizes as poorly conserved. None of these findings support the idea that the voltage paddle sequence is conserved in Kv channels.

While the voltage paddle sequence may not be conserved, its structure and function may still be a common feature of Kv channels. Hydrophobic mutations are one means of testing this idea; as in scanning studies, such mutations typically have large effects only at side chains interacting with other parts of the protein. One study comparing the Shaker and Shaw subfamilies found that a combination of three conservative mutations in this region (V369I, I372L, S376T, or "ILT") shifts the channel's voltage dependence by 120 mV (Smith-Maxwell et al., 1998). A second Shaker study found six hydrophobic

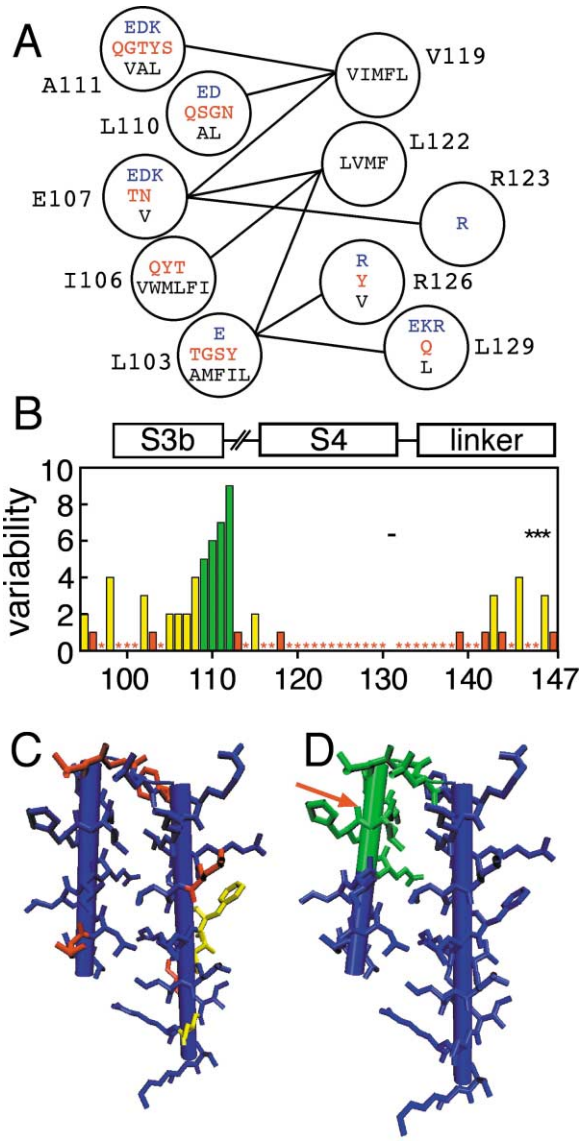

Figure 3. Analysis of the Voltage Paddle

(A) Variability in the key amino acids forming the voltage paddle, using the 18 channels listed in the alignment in Figure 6b of Jiang et al. (2003a). Interacting positions across the paddle interface in the S1–S4 structure were identified as those with atom-atom distances under 4 Å and are shown here connected by lines. Amino acid substitutions at each position are shown in the circles: polar residues are in red, charged residues are in blue, and nonpolar residues are in black. Side chain chemistry does not appear to be conserved in S3b. (B) Sequence variability in the paddle region of Kv1. 84 members of the Kv1 family were obtained by a BLAST hit to Shaker (gi:13432103) and subsequently aligned using ClustalW with default parameters. Variability is defined as the number of different amino acids found at a particular position in the alignment. Amino acid numbers correspond to KvAP as determined from the alignment provided by Jiang et al. (2003a). Red asterisks are absolutely conserved positions. Black asterisks are amino acids in Kv1 channels missing from the alignment to KvAP, and the black dash is an amino acid in KvAP missing in the Kv1 family. (C) Hydrophobic Shaker mutations with large effects on channel electrophysiology. Mutations identified by Lopez et al. (1991) are mapped to the S1–S4 fragment in red, while the ILT mutants are identified in yellow (Smith-Maxwell et al., 1998). None of these positions involve protein-protein interactions. (D) Extreme mutations in the paddle. Deletions of the S3–S4 linker region and portions of the paddle (Gonzalez et al., 2001) are shown in green on the KvAP solo fragment. A significant portion of the paddle can be deleted with only minor changes to channel electrophysiology. The red arrow indicates the point of insertion of the Flag epitope (DYKDDDDK) into Shaker (Shih and Goldin, 1997). Channel expression and function were similar to wild-type.

mutations in this region that shift the voltage dependence by at least 20 mV (Lopez et al., 1991). Remarkably, none of these side chains face the protein-protein interface in KvAP (Figure 3C). Several point to lipid, while others are buried in the Fab binding sites. This suggests that the paddle region has interactions not present in the KvAP models.

The appearance of some of these positions within the Fab binding site raises a vexing question: has the Fab trapped KvAP in a conformation not normally present in functional channels, by binding to a region that would otherwise be interacting with some other part of the channel? This could explain the seemingly paradoxical observation that addition of paddle binding proteins (Fab or tarantula toxins) to the extracellular side of the channel eliminates current, rather than locking the channel open. The authors offer a different explanation, citing the inactivation that normally follows Kv channel opening. Both possibilities are consistent with the inactivation observed in Ruta et al. (2003), but the electrophysiology does not address what sort of structural effects the toxins and Fabs might have on the channel.

Other experiments have examined more extreme alterations in this part of the channel, such as an analysis of a series of deletions in the Shaker S3–S4 linker (Gonzalez et al., 2001). Removal of a segment including 8 residues from the core of the voltage paddle, shown in green in Figure 3D, did not significantly alter channel expression or open probability, indicating that the voltage-sensor undergoes the same motion through the membrane as the wild-type channel. Similar results were seen for seven other mutant channels missing various combinations of residues from within this region. A second study looked at the insertion of the Flag epitope—an eight amino acid sequence with five aspartates, two lysines, and a tyrosine—at sites in the Shaker S3–S4 linker (Shih and Goldin, 1997). Epitope insertion after the equivalent of KvAP L110 (red arrow in Figure 3D), squarely in the middle of the paddle, resulted in channels that had similar expression and physiology to wild-type channels. This Flag epitope could be labeled by extracellular, but not intracellular, antibody staining at resting membrane potential. It is difficult to understand how the channel could accommodate such wholesale manipulation in a critical, core domain without major effects on expression and physiology. These experiments will need to be addressed for KvAP if the claim is to be made that its structure is representative of all Kv channels.

KvAP, Open and Closed

The full KvAP structure presents only a single state of the channel and therefore only part of the picture for understanding how the channel responds to changes in membrane potential. The voltage sensor motion is the subject of the last paper in the series, which examines voltage-dependent changes in the bilayer position of

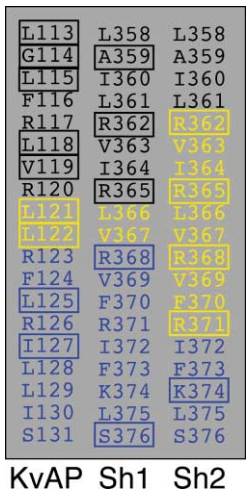

Figure 4. Scanning Mutagenesis Studies of Voltage-Dependent S4 Motion for KvAP and Shaker Channels

KvAP cysteine accessibility study using biotin-avidin (Jiang et al., 2003b); Shaker cysteine reactivity with MTS reagents (Sh1) (Larsson et al., 1996); and Shaker pH-dependent effects of histidine contribution to gating charges (Sh2) (Starace and Bezanilla, 2001). Amino acids in black are only accessible from the outside, those in blue are accessible from the inside, and yellow ones are accessible from either side. Boxed amino acids were tested directly in the cited studies, while the accessibility of unboxed colored amino acids are inferred by us. Residue positions in the same row correspond to the aligned sequences. The biotin and MTS results are indistinguishable, and all three studies center on the same region of S4.

the voltage paddle (Jiang et al., 2003b). MacKinnon and coworkers use a clever series of experiments to measure the depth of a given residue in the bilayer: individual cysteines are introduced and covalently modified with a biotin derivative, which then acts as a molecular dipstick, measuring accessibility to avidin added to either side of the membrane. The data from this paper are also used to corroborate and refine the crystal structures, showing that the pattern of accessibility is consistent with a paddle structure. The most dramatic result is that biotins tethered to two positions, L121 and L122, are accessible to both the inner and outer solutions. This implies that the S4 region is in contact with a significant portion of lipid, which is structurally permissive enough to allow the biotin to be pulled across the membrane.

This study is in some ways similar to Kv channel "cysteine accessibility" studies, which assay the ability of individual cysteines to react with MTS reagents or late transition metal cations (e.g., Larsson et al., 1996). The biotin method has the advantage of being a true accessibility study; MTS studies actually measure cysteine *reactivity*, of which accessibility is just one component (and possibly a minor component; see Shaked et al., 1980). Despite this difference, an alignment of these studies between KvAP and Shaker (Figure 4) shows exact agreement in the accessibility of this portion of the voltage sensor. An analogous Shaker study determined the movement of individual histidines introduced into S4 by examining pH-dependent changes in the gating charge (Starace and Bezanilla, 2001). This is also not a direct measure of accessibility, but rather of the motions of these histidines through the membrane electric field. The region with dual accessibility is larger than in the biotin study but centers on the same part of S4, and the extended boundaries may be due to difficulties inherent in interpreting gating charge variation in terms of side chain position.

While the biotin study gives us a good idea of S4 positions relative to solutions on either side of the bilayer, there are no data about the rest of the channel or the S4 position relative to the pore. The open channel in Jiang et al. (2003b), presented as a working model, keeps the voltage paddle at the channel periphery, moving it up through the membrane and rotating it 90°. As support for the idea that S4 is highly flexible and at the periphery in the open state, they cite a recent report demonstrating that Shaker S4 segments from different subunits can come close enough to each other to form a disulfide bridge between cysteines substituted just before the first arginine (Aziz et al., 2002). Disulfide bridges are typically 6 Å from C_α to C_α (Katz and Kossiakoff, 1986), and the Shaker result is taken to mean that the S4 segments are flexible enough to come together, despite over 40 Å between equivalent KvAP positions in adjacent subunits in the open model. Two other possibilities should be considered. First, the disulfide could be formed in the open state if the ends of the S4 helix move close to each other around the central pore, which would also explain the ability of residues in S4 to form disulfides with positions near either S5 or S6 in the pore region (Gandhi and Isacoff, 2003; Lainé et al., 2003 [this issue of *Neuron*]). A second possibility is that the disulfide is occurring on the periphery between subunits from different channels in the membrane, rather than between subunits from the same channel. It will be important to examine these scenarios more closely in future studies.

The data described here suggest that the relationship between the KvAP structures and other Kv channels remains unclear, and it raises a series of important questions. How has evolution of these channels—such as acquisition of the intra- and extracellular domains necessary for eukaryotic function—affected their structure? Why, if the S4 segment is so flexible, do many of its charged side chains reside in the nonpolar part of the membrane rather than in the water just a few angstroms away, which would be much more energetically favorable? Are the S4 segments of eukaryotic channels as flexible as those of KvAP, or are they constrained by interactions with other parts of the channel not found in KvAP, such as the cytoplasmic T1 domain? And what is the effect of introducing a nonnative binding protein—is there any guarantee of a physiologically relevant channel conformation? The KvAP structures can help us to answer these questions by serving as a starting point, to guide the sorts of experiments described above.

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