The structure of the lipid-embedded potassium channel voltage sensor determined by double-electron–electron resonance spectroscopy

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Abstract

A four-pulse electron paramagnetic resonance experiment was used to measure long-range inter-subunit distances in reconstituted KvAP, a voltage-dependent potassium (Kv) channel. The measurements have allowed us to reach the following five conclusions about the native structure of the voltage sensor of KvAP. First, the S1 helix of the voltage sensor engages in a helix packing interaction with the pore domain. Second, the crystallographically observed antiparallel helix-turn-helix motif of the voltage-sensing paddle is retained in the membrane-embedded voltage sensor. Third, the paddle is oriented in such a way as to expose one face to the pore domain and the opposite face to the membrane. Fourth, the paddle and the pore domain appear to be separated by a gap that is sufficiently wide for lipids to penetrate between the two domains. Fifth, the critical voltage-sensing arginine residues on the paddle appear to be lipid exposed. These results demonstrate the importance of the membrane for the native structure of Kv channels, suggest that lipids are an integral part of their native structure, and place the voltage-sensing machinery into a complex lipid environment near the pore domain.

Keywords: electron spin resonance spectroscopy; potassium channels; voltage-gated; protein conformation

Supplemental material: see www.proteinscience.org

Voltage-dependent potassium (Kv) channels open and close a potassium selective pore in response to changes in the membrane electric potential. The dual functions of selectivity and voltage-dependent gating are conferred by two domains: a pore domain and a voltage-sensing domain, respectively. While the available crystal structures of Kv channels (Protein Data Bank [PDB] entries

1ORS, 1ORQ, 2A0L, and 2A79) all agree with respect to the structure of the pore domain, they depict different voltage sensor structures and domain interfaces (Jiang et al. 2003a; Lee et al. 2005; Long et al. 2005a). The bevy of alternative crystal structures suggest that the two-domain architecture of Kv channels (Murata et al. 2005) and the apparent structural flexibility of their voltage sensor make these proteins challenging targets for structural studies. Detergent solubilization, in particular, seems to perturb the structure of the voltage sensor and disrupt the weak interface between the voltage sensor and the pore (Lee et al. 2005). Despite this distortion, it is expected that individual secondary or tertiary structural elements of the channel can withstand solubilization and

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thus may be used to assemble a native channel structure in a process akin to assembling a puzzle from individual pieces. Long-range distance constraints measured under native conditions can offer insight into the unperturbed membrane protein structure and help provide the necessary constraints to reassemble structural elements that have been displaced during solubilization and crystallization (Skolnick et al. 1997). Electron paramagnetic resonance (EPR) spectroscopy combined with site-directed spin-labeling provides a powerful means to measure distances in reconstituted membrane proteins, and recent advances have expanded its measurable range to inter-subunit distances in multimeric proteins (Pannier et al. 2000; Borbat et al. 2002, 2004; Jeschke 2002; Banham et al. 2006).

In the present study, double-electron-electron resonance spectroscopy (DEER) was used to determine inter-subunit distance distributions between nitroxides introduced throughout KvAP, a tetrameric prokaryotic Kv channel that has been extensively studied both structurally and functionally (Jiang et al. 2003a,b, 2004; Cuello et al. 2004; Ruta and MacKinnon 2004; Lee et al. 2005; Ruta et al. 2005; Schmidt et al. 2006). All measurements were made with KvAP reconstituted into liposomes and in the absence of an applied membrane potential; the channels are therefore presumably in an open-inactivated state. We report results from a total of 16 different sites in the monomer and use the resulting distance distributions to constrain the structure of KvAP. Thirteen of our sites show inter-subunit distance distributions with maxima ranging from ~ 27 Å to ~ 49 Å, whereas the other three sites were determined to be located too far from the symmetry axis of the tetramer for the distances to be within range of the technique as applied here (~ 50 Å). The pore domain data are in excellent agreement with known structures, but the distances obtained for residues in the voltage sensor disagree with those observed in the known crystal structures.

By using short range distance constraints, we show that the antiparallel packing of the voltage-sensing paddle (S3b-S4), a prominent motif of the voltage sensor that contains the critical voltage-sensing arginine residues, is retained in a membrane environment. The paddle can be placed with respect to the symmetry axis, revealing a set of possible native orientations with respect to the pore domain and the membrane. From these placements, it appears likely that all four arginines of the paddle are lipid exposed. The radial distance of the paddle is well constrained by the data and indicates that the paddle is separated from the pore domain by a gap that is sufficiently wide for at least one layer of lipids to penetrate between the two domains. This suggests the possibility that the paddle and the pore interact indirectly through a layer of common boundary lipids.

Results

Inter-subunit distance determination by DEER in reconstituted KvAP channels

When the nitroxide side chain R1 is introduced into the KvAP potassium channel by site-directed spin-labeling, a fourfold symmetric assembly of four magnetically interacting spins with two distinct intramolecular distances is generated (Fig. 1). Different sites in the channel are expected to exhibit different interspin distances primarily because of differences in radial distance from the symmetry axis. Data from three nitroxides (L37R1, L159R1, I130R1) introduced at increasing radial distance from the symmetry axis are shown in Figure 2 to illustrate the DEER experiment. As the R1 probe is introduced at increasing distance from the symmetry axis, the period of oscillation of the dipolar evolution function increases (left panel) and the splitting of the dipolar interaction spectrum narrows (right panel) until the intramolecular signal is lost at a distance of ~ 50 Å. Due to the symmetry of KvAP, there are always two distinct sets of distances (d , $\sqrt{2} \cdot d$) that contribute to the intramolecular dipolar interaction (Fig. 1C). The two distances can be resolved

Figure 1. Site-directed spin-labeling of KvAP. (A) An unpaired electron is introduced into KvAP by mutating a given site in the protein to cysteine and reacting its free thiol with a selective nitroxide reagent. The resulting paramagnetic amino acid is referred to as R1. (B) Topology of the KvAP voltage sensing domain (S1–S4) and sites of the introduced R1 side chain (amino acid positions in bold). The paddle consists of S3b and part of S4 as indicated. (C) Schematic view of the KvAP potassium channel. P is the pore region, and V is the voltage sensor of a single subunit. Four identical subunits are arranged in a fourfold symmetrical fashion around an axis normal to the membrane. The four pore regions together form the pore domain, the functional unit for ion permeation. Spin-labeling at a single site (black circles) generates two sets of inter-subunit distances (arrows) related by a factor of $\sqrt{2}$.

Figure 2. DEER data from three sites located at increasing radial distance from the symmetry axis (L37R1, L159R1, and I130R1). The *left* column shows the inter-subunit dipolar evolution function. The *right* column shows the dipolar interaction spectrum, a weighted sum of Pake functions, obtained by Fourier transformation of the dipolar evolution function. Arrows point to the observed splitting of the dipolar interaction spectrum for site 37.

under favorable experimental conditions, i.e., when a site is located close to the symmetry axis and displays a narrow distance distribution. This is the case for the S1 mutant L37R1, where the dipolar interaction spectrum exhibits two distinct splittings at a distance ratio of $\sim\sqrt{2}$ (arrows in right panel of Fig. 2). In the more typical case featuring a larger radial distance and a broader distance distribution, the diagonal distance manifests itself mainly through increased dampening of the dipolar evolution function and cannot be resolved as a separate distance (L159R1 and I130R1) (Fig. 2). The information originating from diagonal distances is weaker than that originating from adjacent distances because the diagonal distance is longer and its relative weight is smaller (there are two adjacent distances for every diagonal distance in the tetramer) (see Fig. 1C). Nevertheless, the overall distance distribution of the nitroxide can be determined directly in all cases from either the dipolar evolution function (Fig. 2C, left panel) or the dipolar interaction spectrum (right panel) through regularization methods. Regularization methods are particularly advantageous in the present case because they allow for the direct implementation of the channel's intrinsic fourfold symmetry into an otherwise model-free distance analysis (see Supplemental material).

We have determined inter-subunit distance distributions of two peripherally located pore residues and 14 residues located throughout the voltage sensor of KvAP (Table 1). Figure 3 shows the dipolar evolution function (left panels) and their corresponding distance distributions (right panels). It is immediately apparent that the distance distribution observed for residue L37R1 is

uniquely narrow. Whereas broad distance distributions can be broad for a number of reasons, a narrow distance distribution always reflects a high degree of local order of the nitroxide. Consistent with the observed small accessible volume of the unpaired electron, we find that the X-band EPR line shape of L37R1 at room temperature reflects an essentially immobile, ordered state of the nitroxide (Fig. 4). This high degree of ordering of the R1 side chain typically occurs only in cases of tertiary interactions (Langen et al. 2000). In addition to being well ordered, residue L37R1 is also located very close to the symmetry axis. In fact, its radial distance (19.2 Å) is significantly shorter than that of I170R1, the most peripherally located residue of the pore domain (27.9 Å). The short radial distance and very narrow distance distribution of L37R1 therefore indicate that this S1 residue is facing inward and toward the symmetry axis and is being immobilized through tertiary interactions with the pore domain. Assuming helicity of the S1 segment, an assumption for which there is strong experimental support (Jiang et al. 2003a; Cuello et al. 2004; Lee et al. 2005), we can conclude that the middle of the S1 helix is engaged in a close and well-ordered interaction with the pore domain.

Comparison of measured distances with known crystal structures

The accuracy of the DEER distance determination can be evaluated by measuring distances between nitroxides introduced into parts of the structure that are known.

Table 1. Measured maxima of inter-subunit distance distributions

Mutant ^a	Segment ^b	Distance (Å) ^c
L26R1	S1	43.4
L37R1	S1	27.2
V41R1	S1	34.2
Q49R1	S1-S2	39.7
K79R1	S2	48.8
K89R1	S3a	46.8
V98R1	S3a	>50
A104R1	S3b	41.2
A111R1	S3b	49.2
F116R1	S3b-S4	>45
V119R1	S4	41.0
L125R1	S4	>50
I127R1	S4	45.4
I130R1	S4	44.1
L159R1	S5	33.8
I170R1	S5	39.6

^aResidues on the voltage sensing paddle are highlighted in bold.

^bSegments are assigned according to Jiang et al. (2003a).

^cDistance distributions were determined by Tikhonov regularization and the maximum entropy regularization method (MEM) using a tetrameric Pake kernel function (see Supplemental material).

Figure 3. Distance determinations by DEER. The ordinate axes are relative echo amplitude (*left*) and relative population (*right*). The *left* panels show the observed dipolar evolution function (gray) and the MEM fit (black, see Supplemental material). The *right* panels show the distance distribution. The substituted amino acid and its location in the structure are given next to the observed dipolar evolution function. Residues V98R1, F116R1, and L125R1 were determined to be located outside of DEER range and are not shown.

We have done this with KvAP residues L159 and I170, which are located at the extreme periphery of the pore domain and should thus demonstrate the maximal radial dimensions of the pore. To account for the flexibility of the nitroxide side chain (Fig. 1A) and the resulting uncertainty as to the precise position of the unpaired electron, a maximum accessible volume of the electron can be defined for each site by generating rotamer libraries based on the known preferred torsion angles of the nitroxide (Tombolato et al. 2006). The measured distance must then correspond to a distance between any two possible rotamers in the library. The pore domain and the rotamer libraries generated for residues L159R1 and I170R1 are depicted in Figure 5A. The DEER constraints are shown in the form of distance circles with radii equal to the measured adjacent distance (Table 1) divided by $\sqrt{2}$. If the distance circle intersects the cloud of possible electron positions, then the measured distance is in agreement with the structure (see Supplemental material). Figure 5A illustrates that both distance circles indeed intersect the electron cloud of their respective

residue, and both distances are thus in agreement with the structure. The two pore residues also serve to illustrate the relationship between the measured distance distribution and the accessible volume of the unpaired electron based on the local structure surrounding the introduced nitroxide. Figure 5B shows that the maximum of the measured distribution, i.e., the most highly populated distance between nitroxides measured in the DEER experiment, falls clearly within the range of all possible distances expected from the accessible volume of the unpaired electron (horizontal bars). This criterion is even more conservative than the geometric analysis shown in Figure 5A, because it takes into account all possible distances between all possible rotamers and not just distances between individual symmetry-related rotamers (see Supplemental material).

When the above criteria are applied to the voltage sensor, the three alternative crystal structures of the sensor (1ORQ, 1ORS, 2A0L) (Jiang et al. 2003a; Lee et al. 2005) cannot be brought into agreement with the measured distances. In other words, no orientation with

Figure 4. Room temperature spectra of selected mutants at X-band. The spectra are grouped according to decreasing mobility. Two dashed lines are drawn at the hyperfine maxima of L37R1 to guide the eye. Q49R1 is the most mobile site studied and is located in the S1-S2 loop, A111R1 and A104R1 show spectra of intermediate mobility typical for surface sites on α -helices, and L37R1 is the most immobile site studied.

respect to a fourfold axis exists for any of the structures such that the most commonly observed distance is within the expected distance range at all positions. The most straightforward conclusion of this finding is that none of the three known crystal structures reflects the structure of the voltage sensor domain as it exists under these experimental conditions. One of the failed placements of the isolated voltage sensor structure (1ORS) is shown in Figure 6. In this particular placement, nine measured distances are in agreement with their residues' expected distance (positions indicated by gray C α spheres), while five distances are not (colored rotameric clouds and distance circles).

The voltage-sensing paddle is a structural element of the membrane-embedded channel

The number of constraints that can be obtained by DEER is insufficient to independently determine the native structure of KvAP. We therefore sought to determine whether structural elements of the voltage sensor were maintained in the known crystal structures that would allow their placement with respect to the symmetry axis using the measured distance constraints. We started with the most prominent structural element defined by crystallography of the KvAP voltage sensor: the S3b-S4 paddle (Fig. 1B), a helix-turn-helix motif that contains the critical voltage-sensing arginine residues (Jiang et al. 2003a). The paddle appears to be a stable motif in detergent since it has been consistently observed in all known structures (Jiang et al. 2003a; Lee et al. 2005). In addition, mobility and accessibility studies of paddle residues have confirmed the overall secondary structure

of the S3b-S4 segment under native conditions (Cuello et al. 2004). In order to determine whether the characteristic antiparallel packing of the S3b and S4 helices of the paddle is conserved in a membrane environment, we introduced pairs of nitroxides into the paddle at positions expected to show strong magnetic dipolar interaction and measured their short-range intra-subunit distance using conventional continuous wave EPR techniques (Fig. 7; Gross et al. 1999). The limited range of this technique (up to ~ 20 Å) ensures that inter-subunit interactions are negligible. The spectra of the two double mutants demonstrate the line shape broadening expected of a close dipolar interaction between nitroxides, consistent with the existence of the antiparallel paddle structure under native conditions.

The paddle substructure can thus serve as a structural scaffold for introducing the nitroxide side chain and can be placed in a fourfold symmetrical arrangement that simultaneously fulfills all DEER distance constraints.

Figure 5. DEER distance constraints of sites located in the pore domain. (A) The KvAP pore domain (PDB entry 1ORQ) is shown as black C α traces. The possible positions of the unpaired electron in the nitroxide are shown as spheres together with the radial distance circle determined by DEER. L159R1, 23.9 Å (red); I170R1, 28.0 Å (blue). Note that the distance circles (more accurately distance cylinders) pass through the rotameric clouds in both mutants in agreement with the crystal structure. (B) The measured distance distributions are compared with the expected distance distributions based on possible rotamers. (Left) L159R1; (right) I170R1. The mean (μ), the first, and the second standard deviation (σ) of the calculated inter-subunit distance distributions are shown above the measured distance distributions. Note that the maximum of the measured distribution lies within 2σ of the mean of the calculated distance distribution in both mutants.

Figure 6. Placement of the isolated voltage sensor (PDB entry 1ORS) fails to fulfill all DEER constraints. The voltage sensor and the pore domain are shown as black and gray C α traces, respectively. The α -helical elements of the voltage sensor are indicated (Jiang et al. 2003a). The relative position of the pore domain is arbitrary. In the orientation of the voltage sensor shown (green orientation of Fig. 9B), five of 14 distance constraints are violated, i.e., the rotamer cloud does not overlap the distance circle. The possible positions of the nitroxide and the distances circle for each mutant are shown as in Figure 5. L26R1 (S1) 30.7 Å (red), L37R1 (S1) 19.2 Å (blue), Q49R1 (S1-S2) 28.1 Å (green), K79R1 (S2) 34.5 Å (cyan), K89R1 (S3a) 33.1 Å (magenta). The remaining nine constraints were fulfilled (gray C α carbons with rotameric clouds and distance circles left out for clarity).

Figure 8 illustrates the results from the same conservative analysis that previously failed with residues distributed throughout the voltage sensor. Any trial position of the paddle, consisting of an orientation and a radial distance from the symmetry axis, was accepted if all distance constraints were simultaneously fulfilled at all sites. Only two small sets of possible paddle orientations are consistent with the measured distances. These two sets are symmetry-related to each other by a twofold rotation around an axis perpendicular to the symmetry axis, corresponding to “up” and “down” orientations of the pore domain, and are thus equivalent solutions. Figure 8 shows paddle orientations obtained using two different stringencies on the distance constraints. In panel A, only the maximum of the observed distance distribution is required to fall within the range of possible inter-residue distances at all sites. Sixteen such solutions were randomly chosen and arranged around the pore structure to demonstrate the range of possible solutions and their relationship to the pore. In panel B, the stringency of the analysis was raised by requiring distances immediately flanking the maximal distance to be within range as well, thus taking into account that a given peak distance in a symmetric protein is only plausible if alternative rotamers can account for flanking distances. The increase in stringency leads to the elimination of borderline acceptable solutions and narrows the set of possible orientations (Fig. 8B). Four features of all paddle solutions are noticeable. First, the radial distance of the paddle to the symmetry axis is highly constrained by the data, and increasing stringency further constrains it (panel A:

35.8 ± 2.1 Å, panel B: 36.4 ± 1.3 Å). Second, the paddle is not packed tightly against the pore domain; instead there is a gap between pore domain and paddle (specifically the S4 segment) independent of the paddle’s rotational placement. Third, the orientation of the paddle is preferentially tangential to the pore domain in the sense that its orientation exposes one face of the paddle toward the pore domain while exposing the opposite face outward and toward the lipid bilayer. A tangential orientation of the paddle is in excellent agreement with independent accessibility data obtained by site-directed spin-labeling that have shown the paddle to be located in an anisotropic environment under near-native conditions (Fig. 9A; Cuello et al. 2004). Fourth, the placement of the paddle around an axis perpendicular to the symmetry axis is poorly constrained (Fig. 9B). Rotation of the paddle around this axis has only little effect on inter-subunit distances, and it is therefore to be expected that the resolution in this dimension is poor. The results thus point to a paddle located at a tightly constrained distance from the pore domain, facing it in a tangential orientation, but with unresolved rotational and translational placement.

Figure 7. Test of the antiparallel packing of the paddle using short-range EPR distance constraints. (A, left) The paddle (PDB entry 1ORS) is shown as a ribbon diagram. The two introduced nitroxides (I106R1, L122R1) and the four critical arginines (R117, R120, R123, R126) are shown in stick representation. Double arrows indicate the separation of the unpaired electrons between two arbitrary nitroxide rotamers. If the two helical segments of the paddle pack against each other in an antiparallel fashion under native conditions then a strong dipolar interaction should be observed between two nitroxides introduced into the paddle. (Right) First derivative absorption EPR spectra of the double mutants are shown with (red) and without (black) dipolar interaction. The noninteracting spectra were obtained by dilution of the magnetic interaction using a mixture of a paramagnetic label (R1) and a nearly isosteric diamagnetic analog (R1') (Gross et al. 1999). (B) L119R1-G108R1 double mutant. Both mutants show clear evidence of line broadening consistent with strong dipolar interaction and hence short distance.

Figure 8. Stereoviews of paddle solutions using different stringencies on the distance constraints. Paddle orientations are shown as colored C α traces with the critical arginine positions marking the S4 helix. (A) The maximum of the distance distribution is required to be within 2 SD of the mean of the expected distance distribution for all sites. Sixteen solutions were randomly selected and placed around the pore domain. (B) Same as in A, but a 2 Å wide band centered at the peak distance is required.

Discussion

DEER distances and their use as structural constraints

Two different strategies can be followed to incorporate DEER distances into a structural analysis. Either the analysis can attempt to determine the most likely nitroxide rotamers giving rise to the measured distance distribution, or it can take into account all possible rotamers. The first methodology depends critically on the algorithm that selects rotamers (Sale et al. 2005), while the second methodology sacrifices precision to achieve inclusiveness. Because energy differences between rotamers can be small and difficult to quantify, we have chosen the second methodology but have added some sensible additional constraints due to the fourfold symmetry of the system. Distance distributions between nitroxides are typically quite broad (on the order of ~ 10 Å) due to the flexibility of the nitroxide side chain (Fig. 1A), (Altenbach et al. 2001). If the data were perfect, then the entire measured distance distribution could be used as a structural constraint on the nitroxide side chain since every populated distance in the distribution would have to

correspond to a feasible side chain structure. However, limitations in the data, both in terms of signal-to-noise and duration of the observed dipolar evolution, tend to broaden the measured distance distributions. Since the maximum of the distance distribution is thus much better constrained by the data than its shape (Jeschke et al. 2004), only this distance and its immediately flanking distances are used as constraints in the present structural analysis. By taking into account all possible distances between all possible rotamers yet requiring only that the best measured distance be feasible at any given site, the analysis is rendered inherently conservative. A distance constraint is thus said to be fulfilled if two nitroxide side chains can be independently positioned in the structure so that the distance between their unpaired electrons matches the measured distance. However, the presence of symmetry substantially increases the strength of the distance constraint in the present case because the measured distance distribution arises through interactions between four symmetry-related identical rotamer distributions. In the context of mobile and thus disordered nitroxides (Fig. 4), it follows that the rotamers giving rise

Figure 9. A paddle orientation consistent with accessibility data and distance constraints. (A) Accessibility data mapped onto the voltage-sensing paddle. Two views of the paddle as a ribbon diagram with the critical arginines shown in stick representation. (Left) A side view is shown corresponding to a view from the symmetry axis. The C α -C β bonds (or the C α carbon for glycine residues) are shown in three different colors according to EPR collision data (Cuello et al. 2004). Blue residues have a higher rate of collision with NiEDDA than with oxygen. These residues reside in a polar environment. Yellow residues have a higher rate of collision with oxygen than with NiEDDA. These residues reside in an apolar environment. Gray residues have comparable collision rates for the two species (within a factor of two). These residues are in an intermediate environment. Note the hydrophilic (blue) character of the face above the arginines and the hydrophobic (yellow) character below the arginines. (Right) A back view of the paddle is shown, corresponding to a view from the lipid bilayer. Note the clear hydrophobic (yellow) character of the entire face. (B) Three paddle orientations consistent with DEER constraints are shown. The red and blue solutions represent extreme orientations, while the green solution represents an intermediate solution. The paddle is shown as a colored C α trace for two subunits, and the pore domain is shown as a gray C α trace in an arbitrary position with respect to the symmetry axis (both rotation and translation). Note that the paddle is unconstrained in a way that can be roughly represented by a rotation around an axis perpendicular to the symmetry axis.

to the most highly populated distance are constrained to a subset of rotamers that do not occupy the extreme edge of the accessible volume (see Supplemental material). This symmetry-based constraint was incorporated into the analysis by requiring that the measured distance fall within the expected range of nonextreme rotamers, i.e., within 2 SD from the mean of all possible distances (Fig. 5B). Local nitroxide disorder and the presence of symmetry thus allow an analysis that takes into account all possible nitroxide rotamers to be made more stringent through the exclusion of extreme rotamers, and the re-

quirement that distances immediately flanking the peak distance must also be possible. Figure 5B illustrates the conservative nature of this analysis when applied to a region of known structure.

The structure of the voltage sensor

DEER data provide sparse yet highly constraining structural information about a protein and it is straightforward to test a known structure with only a few distances (Fig. 5). In the present case, there already exists a near consensus in the field that the two known crystal structures of full-length KvAP depict nonnative conformations of the voltage sensor, and the present data further support this view (Cohen et al. 2003; Jiang et al. 2003a; Laine et al. 2004; Bezanilla 2005; Richardson et al. 2006). It may be that the channel requires a membrane to correctly position its two domains and that the weak interdomain interactions are lost in the absence of a lateral force imposed by the membrane (Lu et al. 2001). However, the high degree of structural plasticity observed among the known voltage sensor structures supports the notion that not only the pore-sensor interaction but the voltage sensor itself may be vulnerable to solubilization (Cuello et al. 2004; Lee et al. 2005). Nevertheless, it is often implicitly assumed that the structure of the isolated voltage sensor (PDB entry 1ORS) approximates a native state (Cohen et al. 2003; Cuello et al. 2004; Ruta et al. 2005; Sands et al. 2006). The present data, however, are inconsistent with this assumption, at least for the state of KvAP present in these experiments, since no orientation of the crystal structure could account, even approximately, for the measured inter-subunit distances. The suggestion that all known crystal structures of the voltage sensor solved in detergent may be nonnative is reasonable in light of the evolutionary origin and function of this domain (Murata et al. 2005; Ramsey et al. 2006; Sasaki et al. 2006). Voltage sensors are specialized membrane proteins that can alter their structure in response to changes in membrane potential and act like molecular switches. As a result of this membrane adaptation, the native structure of the voltage sensor may well exist only in a membrane environment.

The available distance constraints cannot uniquely determine the native structure and orientation of the complete voltage sensor, but they may be used to adapt a nonnative structure to a near-native state. In order to evaluate the degree to which the isolated voltage sensor structure is at variance with the distance constraints, the structure is placed so that its paddle conforms to the DEER data measured for paddle sites (Fig. 6). In this placement, we observe a substantial discrepancy between the location of non-paddle residues and their measured radial distances. Specifically, the S1-S2 loop is in steric conflict with the pore domain irrespective of its rotational

placement and must be moved further away from the symmetry axis, whereas the S2-S3 loop must be moved closer to the symmetry axis. The S1 helix in turn must be packed against the pore domain for the entire sensor to be in compliance with the distance constraints. Conceptually, the simplest modification that achieves rough agreement with the data while maintaining large aspects of the sensor structure would be to join the short S3a and S3b helices of the voltage sensor into a single S3 helix analogous to the transmembrane S3 helix observed in the structure of Kv1.2 (Long et al. 2005a). This modification is attractive because it removes a major structural difference between two functionally equivalent structural elements of two homologous proteins, but many other structural rearrangements are equally possible and consistent with the data.

The S1 helix packs against the pore domain

Three important features of the native voltage sensor structure are strongly constrained by the present data: (1) the presence of a packing interaction between the S1 segment of the sensor and the pore domain, (2) the compact antiparallel helix-turn-helix structure of the paddle, and (3) the orientation of the paddle with respect to the membrane and the pore domain. The proposed close packing interaction between S1 and the pore domain rests predominantly on a single finding: the identification of a close, highly ordered, and immobile R1 side chain in the middle of S1. The radial proximity of S1 is further supported by the generally close distances at sites 41 (top of S1) and 49 (S1-S2 loop). In addition, a packing interaction of S1 with the pore domain is in excellent agreement with nitroxide scanning results that show generally damped apparent side-chain mobility combined with low oxygen accessibility of S1 residues consistent with a helix packing interaction (Cuello et al. 2004). Interestingly, both crystal structures of full-length KvAP feature very similar packing interactions between a helical S1 segment and the pore domain (Fig. 10) but depict the S1 helix as being oriented in the opposite direction compared with the orientation determined under native conditions (Cuello et al. 2004). The adjacent inter-subunit distances between inward-facing S1 residues involved in the packing interaction in both crystals range from ~26 to 33 Å (C β) and are thus very similar to the measured distance at site 37 (~27 Å). The interaction surface of the S1 helix in the crystal is made of residues Leu36, Leu37, Ile40, Val43, Val44, and Thr47, and it therefore appears that the native interaction surface of S1 is maintained in the nonnative crystal structure. During solubilization and crystallization of the channel, the native interaction is apparently first disrupted and then later re-established using the same

Figure 10. Helix packing based on “ridges in grooves” models between S1 and the pore domain. The S1, S5, and S6 helices are shown schematically with two ridges/grooves (solid and dotted lines). In the crystal structures of full-length KvAP, the dotted ridges of S1 fit into the dotted grooves of the pore (crystal packing, *left* panel). There are two likely native packing interactions: The dotted ridges of S1 fit into the dotted grooves of the pore (packing 1, *middle* panel), or both ridges of S1 fit into both grooves of the pore (packing 2, *right* panel).

face of the S1 helix but with the helix running in the opposite direction (Fig. 10). It is evident that the structure of the voltage sensor must be significantly perturbed from its native state to accommodate this inverted helix packing interaction.

Helix packing interactions can be understood on the basis of a “ridges in grooves” model that predicts how α -helices pack against each other (Branden and Tooze 1999). If these simple packing rules are applied to the packing of S1, then two alternative packing interactions emerge as likely candidates for the native structure (Fig. 10): a first orientation in which the S1 helix occupies the same groove as in the crystal structure but is running in the opposite direction, and a second orientation along the alternative groove not occupied in the crystal. The two alternative helix packing interactions predict very different angles of the S1 helix with respect to the membrane. Although the present data are consistent with both packing interactions, previous accessibility studies clearly favor the near-normal helix orientation (native packing 2) due to the observation that the spacing between highly water-accessible sites (marking the approximate width of the membrane) in S1 is 27 residues (Cuello et al. 2004), a value consistent with a near-normal (~20°) but too small for a highly tilted (~50°), trans-membrane helix.

The orientation of the paddle in lipid bilayers

The antiparallel helix-turn-helix motif of the paddle is observed in all crystallographic studies of KvAP (Jiang et al. 2003a; Lee et al. 2005), and the secondary structure of the motif has been confirmed under native conditions (Cuello et al. 2004). The short-range dipolar interaction observed between R1 side chains introduced into the paddle (Fig. 7) strongly supports the notion that the

paddle is a native motif of the membrane-imbedded voltage sensor. While the gross structure of the paddle is thus confirmed, the EPR constraints, based on the presence of two independent nitroxide rotamer distributions, cannot exclude antiparallel paddle structures that are slightly different from those observed crystallographically. However, in light of the highly reproducible crystallographic structure of the paddle (with root-mean-square deviations of 0.9 Å and 0.6 Å for 2A0L/1ORS and 1ORQ/1ORS, respectively), it appears warranted to use the paddle substructure of the isolated sensor (1ORS), being the best defined in terms of resolution, as a scaffold to place the nitroxide side chains and determine the accessible volume of the unpaired electron at each site. These placements allow the paddle to be oriented with respect to the symmetry axis using only distance constraints. Moderately increasing the stringency of these constraints yields a well-defined paddle orientation containing an inherent uncertainty in one orientation (Fig. 8). Not included in this geometric analysis is an important and strong constraint on the paddle position, namely, the simple fact that the paddle must connect to both the S3a and S5 segments of the channel. There is strong experimental evidence that both the S2-S3 loop and the S4-S5 connector are exposed to the intracellular side of the membrane (Cuello et al. 2004). In addition, the intervening residues between these intracellular points and the paddle are short and predominantly α -helical (Jiang et al. 2003a; Cuello et al. 2004; Lee et al. 2005), limiting the range of these connections. Close inspection of the solutions allowed by the conservative initial criterion applied to the DEER distances (Fig. 8A) and how these solutions change upon tightening of the constraints (Fig. 8B), reveals that the solutions eliminated from the pool as being borderline acceptable in terms of distance, also fail the connectivity test. The connectivity argument, depth constraints based on biotin capture (Jiang et al. 2003b; Ruta et al. 2005), accessibility constraints based on collision rates with paramagnetic species (Cuello et al. 2004), and the present distance constraints together all point to a single consistent orientation of the paddle. This orientation can be described as being more vertical than horizontal and roughly bracketed by the red and green solutions depicted in Figure 9B. Notably, the orientation of the paddle (S4 segment) observed in the recent crystal structure of Kv1.2, a eukaryotic potassium channel, lies outside the allowed range determined here for KvAP (Long et al. 2005a). Further experiments are required to determine whether this difference between KvAP and Kv1.2, as well as the apparent difference in the packing of S1, are genuine differences between native structures of homologous proteins. Alternatively, it appears that these differences can be reconciled by a $\sim 90^\circ$ in-plane rotation of the

Kv1.2 voltage sensor followed by its repacking against the pore domain.

A gap exists between the paddle and the pore domain

The DEER distances constrain only the orientation and radial distance of the paddle with respect to the symmetry axis. The relative position of the paddle around and along the symmetry axis, on the other hand, remains entirely unconstrained. Interestingly, the distance of the paddle from the symmetry axis is such that there is always a gap, or crevice, between the two structures, independent of their relative rotation and/or translation. The actual width of the gap is poorly determined and depends on the precise location of the paddle with respect to the pore. The gap is narrowest if the paddle is located near the extracellular surface of S5, where the pore domain is largest in terms of its radial dimension. A native position of the paddle near S5 appears likely based on cross-linking experiments between the voltage sensor and the pore on KvAP (Lee et al. 2005), the requirement that S4 connect to S5, and the overall structural similarity between KvAP and Kv1.2 (Long et al. 2005a,b). However, even if the paddle is placed near S5, the gap retains a minimum width on the order of 5–6 Å (Figs. 8, 9B) and is thus sufficiently wide for at least one layer of lipids (Engelman 1971), some water, or a combination of both to penetrate between the two domains. It thus appears that at the level of the paddle there is no direct interaction between the two domains and that the environment experienced by inward-facing residues on the paddle, including two of the four critical arginines (Arg123 and Arg126), is likely non-proteinaceous. The other two critical arginines (Arg117 and Arg120) on the paddle project sideways into the bilayer, and their environment is therefore highly dependent on the precise orientation of the paddle and its immersion depth with respect to the membrane (Fig. 9B). The orientation of the paddle thus determines the position and projection of the critical arginine residues, while its distance from the symmetry axis determines their degree of interaction with the pore domain.

The presence of a gap between the two domains is an unexpected finding that has important implications for the structure and function of the channel, as highlighted by the fact that two of the four critical arginines of the paddle project into it (Arg123 and Arg126) (Fig. 9A). Accessibility studies point to the presence of a water-lipid interface in the gap as the environment experienced by gap-exposed residues is polar above Arg123 and Arg126 and apolar below them (Fig. 9A; Cuello et al. 2004). The two arginines would therefore occupy an intermediate position near the water-lipid boundary in the gap and be located in an electrically external environment.

The other two arginines (Arg117 and Arg120) can similarly be positioned at the water–lipid interface as they project sideways into the bilayer. In these orientations, all arginines are positioned to hydrogen bond with phosphate moieties of the membrane as arginines have been shown to do in other membrane protein structures (Hunte 2005). Indeed, such an arginine–phosphate interaction was recently proposed based on functional studies using lipids with altered structure (Schmidt et al. 2006). The placement of the critical arginine side chains thus appears both energetically feasible and consistent with functional studies (Jiang et al. 2003b; Ruta et al. 2005; Schmidt et al. 2006). Although the present data cannot identify the gap filler, a partially lipid-filled gap is an attractive possibility because it would provide straightforward explanations for the apparent modular behavior of the voltage sensor and the pore domain (Lu et al. 2001), the observed functional significance of lipid-modifying enzymes and lipid structure on channel gating (Ramu et al. 2006; Schmidt et al. 2006), the measured collision rates of gap-exposed residues with different paramagnetic species (Cuello et al. 2004), the observed detrimental effect of detergent solubilization on channel structure, and the improved nativity of structures solved in the presence of lipids or lipid-like detergents (Lee et al. 2005; Long et al. 2005a). In this view, detergent solubilization leads to the removal of a critical structural component from the channel. In addition, the presence of lipids in the gap could play a major role in the mechanism of voltage-dependent gating because the close interaction of these lipids with the surrounding protein has the potential to substantially alter the profile and width of the transmembrane electric field in the gap (Freites et al. 2005). Lipids may thus not only be an integral part of the structure of Kv channels, but may also constitute an important component of their gating mechanism.

Materials and Methods

Protein expression, purification, labeling, and reconstitution

KvAP was overexpressed and purified as described (Ruta et al. 2003) with minor modifications. A DNA sequence coding for KvAP was synthesized and subcloned into the pQE60 expression vector (Qiagen). Site-directed mutagenesis was performed with QuikChange (Stratagene) and verified by sequencing. *E. coli* were transformed and grown in a 2 L fermentor (BioFlo 110, New Brunswick Scientific), induced with IPTG in the presence of 10 mM BaCl₂, and cultured. After sonication, KvAP was solubilized with 40 mM of decylmaltoside (DM) in lysis buffer (20 mM Tris, pH 8.0, 100 mM KCl), loaded on a cobalt resin (Talon, Clontech), spin-labeled overnight with a 10-fold molar excess of MTSL ([1-oxy-2,2,5,5 tetramethyl- Δ^3 -pyrroline-methyl] methanethiosulfonate) or a 1:3 mixture (Gross et al. 1999) with (1-acety-2,2,5,5 tetramethyl- Δ^3 -pyrroline-3-

methyl) methanethiosulfonate (both Toronto Research), and eluted with 500 mM imidazole. KvAP was further purified by gel filtration on Superdex 200 in lysis buffer with 5 mM DM and reconstituted on Sephadex G-50 (both GE Healthcare) into POPC:POPG (4:1) liposomes (Avanti Polar) at a molar lipid:protein ratio of $\sim 1000:1$ by the method of Green and Bell (1984). Proteoliposomes were concentrated by centrifugation and resuspended to a KvAP concentration of $\sim 200 \mu\text{M}$ with a solution of 50 mM Tris (pH 7.4), 150 mM KCl, and 25% glycerol. All data were obtained with spin-labeled KvAP channels reconstituted in lipid vesicles in the absence of an applied membrane potential, and thus KvAP is expected to be in an open, inactivated conformation.

EPR measurements and determination of distance distributions

CW EPR spectra were recorded at room temperature on an X-band EMX spectrometer fitted with an ER4119HS resonator (Bruker Biospin) at a scan width of 200 Gauss. Samples with diluted magnetic dipolar interaction were prepared by reacting the double mutants with a mixture of two labels: a paramagnetic label and an approximately isosteric diamagnetic analog (Gross et al. 1999). DEER samples were flash-cooled in liquid nitrogen, and the data were recorded at 50 K on an Elexsys-E580 spectrometer fitted with an ER4118X-MS2 resonator (Bruker Biospin). Data were acquired using a four-pulse protocol (Pannier et al. 2000) with an 8-ns $\pi/2$ and two 16-ns π observe pulses separated by a 16-ns π pump pulse. The pump frequency was set to coincide with the center line of the nitroxide spectrum and the observer frequency to the low field line. Inter-subunit distance distributions were determined using established model-free distance domain Tikhonov and maximum entropy regularization methods in conjunction with a two-dimensional background model (Jeschke et al. 2004; Chiang et al. 2005a,b).

Structural analysis

A fundamental difficulty in analyzing DEER data in terms of structure is due to the fact that the measured inter-subunit distance distributions arise from unknown nitroxide rotamer distributions. Because energy differences between side chain rotamers can be small, the present analysis does not attempt to predict the most likely nitroxide rotamer for any given site (Sale et al. 2005) but instead takes into account all energetically feasible nitroxide rotamers (Tombolato et al. 2006). By sampling the entire accessible rotameric space of the nitroxide simultaneously at all sites, the structural analysis is inherently conservative. A distance constraint is said to be fulfilled if the measured distance falls within a range of distances that can be reconciled with possible nitroxide rotamers. All inter-subunit distance distributions originate from interactions between symmetry-related nitroxide rotamer distributions. The maximum of the inter-subunit distance distribution occurs at the most common inter-subunit distance and must thus originate from a subset of common, namely, not extreme, rotamers. This additional symmetry constraint can be incorporated into the analysis by requiring that the maximum of the measured distance distribution fall within 2 SD of the mean of a distance distribution calculated from all possible nitroxide rotamers. At higher stringency, a distance constraint was said to be fulfilled if a 2 Å wide band of distances centered at the maximum distance can similarly be reconciled with possible nitroxide rotamers,

thus taking into account that a maximum can only be observed if both shorter and longer distances are also possible. The coordinates of the paddle locations shown in Figure 8B are available from the authors upon request.

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