Voltage-sensing domains (VSDs) play diverse roles in biology. As integral components, they can detect changes in the membrane potential of a cell and couple these changes to activity of ion channels and enzymes. As independent proteins, homologues of the VSD can function as voltage-dependent proton channels. To sense voltage changes, the positively charged fourth transmembrane segment, S4, must move across the energetically unfavorable hydrophobic core of the bilayer, which presents a barrier to movement of both charged species and protons. To reduce the barrier to S4 movement, it has been suggested that aqueous crevices may penetrate the protein, reducing the extent of total movement. To investigate this hypothesis in a system containing fully functional channels in a native environment with an intact membrane potential, we have determined the contour of the membrane-aqueous border of the VSD of KvAP in Escherichia coli by examining the chemical accessibility of introduced cysteines. The results revealed the contour of the membrane-aqueous border of the VSD in its activated conformation. The water-inaccessible regions of S1 and S2 correspond to the standard width of the membrane bilayer (28 Å), but those of S3 and S4 are considerably shorter (40%), consistent with aqueous crevices pervading both the extracellular and intracellular ends. One face of S3b and the entire S3a were water-accessible, reducing the water-inaccessible region of S3 to just 10 residues, significantly shorter than for S4. The results suggest a key role for S3 in reducing the distance S4 needs to move to elicit gating.

VSDs to play critical roles in a wide range of physiological processes, including the excitability of neuronal, cardiac, and muscle cells and the secretion of hormones and neurotransmitters. A recent study showed that the role of VSD is not restricted to ion channels but extends to other proteins. In Ciona intestinalis, an ascidian, the VSD has been found to be coupled to a phosphatase enzyme (CiVSP), where it regulates the activity of the enzyme in a voltage-dependent manner. Remarkably, it seems that VSDs are not merely integral regulatory components of channels and enzymes, but can function independently. For example, Hv1, a homologue of the VSD found in the human genome, is able to conduct protons by itself, and this function has a physiological role in nonexcitable tissues, such as testis, intestine, and blood cells.

VSDs comprise four transmembrane domains, S1–S4. In voltage-gated ion channels, four VSDs are covalently linked to a central pore domain, made from the tetrameric assembly of S5–S6 helices (2–4). The S4 helix has six to seven conserved positively charged residues (arginine/lysine), of which the first four appear to contribute to the voltage sensing function (3, 4). The crystal structure of an isolated VSD from the bacterial voltage-gated potassium (Kv) channel, KvAP, has been solved (9). Although the structure bears a remarkable resemblance to the VSD in the more recently solved rat Kv1.2 structure (10), its structure differs significantly from the VSD in the full-length KvAP (9, 11). Interestingly, mutation of some S4 arginines to less bulky amino acids confers proton conducting function on the VSD (12–14). In voltage-gated Na+ channels such mutations lead to proton leak and underlie hypokalaemic periodic paralysis (15). Despite these breakthrough studies, the molecular basis of how the VSD senses changes in membrane potential and conducts protons remains unclear.

It is generally accepted, however, that in response to changes in membrane potential, the top four S4 arginines (spanning a distance of 13.5 Å) move across the hydrophobic core of the bilayer (2–4, 16). However, the mechanism by which this is accomplished remains highly controversial (3, 4, 17, 18). Two main divergent models have emerged: a “paddle model” and a “focused-field model.” According to the paddle model, S4 and the C-terminal end of S3, S3b, form a helix-turn-helix structure (termed a paddle), which moves as a rigid body across the membrane by 15–20 Å during gating (16, 19). Such large motions seem to be consistent with the large charge translocation reported in eukaryotic channels. However, the paddle model is based exclusively on avidin capture studies of KvAP, and it contradicts the focused-field model, which was developed from several lines of evidence on eukaryotic channels, all indicating a...
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much smaller (≤5 Å) S4 translation (3, 12, 20–22). To account for the large charge (≈13 e) translocation with only a small S4 translation, it was proposed that the hydrophobic core of the bilayer is thin in the vicinity of S4, with large aqueous crevices penetrating the bilayer from both the extracellular and cytosolic phases (3, 4, 12, 17). However, such aqueous crevices are not discernible in the crystal structures of KvAP (9, 11). Given the significant homology between KvAP and eukaryotic channels (9), it is difficult to envisage how the structure of KvAP could differ from that of eukaryotic channels. Indeed, modeling studies suggest that KvAP is structurally similar to rat Kv1.2 (10).

Several studies have examined the topology of KvAP VSD in a bilayer. Molecular dynamics simulation studies suggest that the bilayer is compressed in the vicinity of S4 and that this compression is brought about by a network of interactions between S4 arginines, lipid phosphates, and water molecules (23, 24). In an EPR study, Perozo et al. (25) examined the accessibility of spin labels attached to different sites of VSD to O2 and nickel(II)-ethylene-NN,N-diaminediacetic acid to determine the lipid- and water-exposed positions, respectively; they reported both water- and lipid-accessible regions of VSD. MacKinnon and co-workers (16, 19) introduced biotin at different positions of KvAP and examined their accessibility to avidin. In all these studies, synthetic lipids were used to reconstitute the channels into bilayers or vesicles, and therefore they do not endorse the conformation of the channel in its native environment. This is because lipids in a native membrane are highly heterogeneous, and their effect on membrane deformation in the vicinity of S4, and hence the configuration of aqueous crevices is likely to be quite different from that of synthetic lipids. Indeed, studies by MacKinnon and co-workers showed that the conformation (11) and voltage-dependence properties (26) of KvAP vary with the nature of the lipid into which they are reconstituted. Moreover, unlike most other protein domains, VSDs are highly flexible, and their conformation is highly dependent on the membrane potential. Thus, it is unclear to what degree native conformation is preserved during the crystallization and reconstitution experiments.

We therefore set out to determine the topology of KvAP in live Escherichia coli, where the channel is in its native environment. For this, we developed a highly sensitive site-directed cysteine accessibility method. The method relies on in vivo radiolabeling of cysteine mutants, reaction of water-exposed cysteines with a thiol modification reagent, and detection of vibrational energy transfer (VET) using EPR spectroscopy. Perozo et al. (25) examined the accessibility of spin labels attached to different sites of VSD to O2 and nickel(II)-ethylene-NN,N-diaminediacetic acid to determine the lipid- and water-exposed positions, respectively; they reported both water- and lipid-accessible regions of VSD. MacKinnon and co-workers (16, 19) introduced biotin at different positions of KvAP and examined their accessibility to avidin. In all these studies, synthetic lipids were used to reconstitute the channels into bilayers or vesicles, and therefore they do not endorse the conformation of the channel in its native environment. This is because lipids in a native membrane are highly heterogeneous, and their effect on membrane deformation in the vicinity of S4, and hence the configuration of aqueous crevices is likely to be quite different from that of synthetic lipids. Indeed, studies by MacKinnon and co-workers showed that the conformation (11) and voltage-dependence properties (26) of KvAP vary with the nature of the lipid into which they are reconstituted. Moreover, unlike most other protein domains, VSDs are highly flexible, and their conformation is highly dependent on the membrane potential. Thus, it is unclear to what degree native conformation is preserved during the crystallization and reconstitution experiments.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology—**KvAP (gi:14601099) was subcloned into the pET28a inducible bacterial expression vector (Novagen). The native cysteine at position 247 was substituted with a serine to generate the cysteine-less KvAP. Cysteine mutations were introduced by the QuickChange site-directed mutagenesis method (Stratagene).

**Selective Radiolabeling of KvAP—**KvAP was selectively radiolabeled using a method described previously (27). Briefly, E. coli BL21(DE3) cells harboring pET28a-KvAP were grown in 5 ml of 2YT medium (1.6% Tryptone, 1% yeast extract, 0.5% NaCl) at 37 °C (150 rpm) to an A600 of 0.4. Cells were pelleted, resuspended in 5 ml of M9 minimal medium supplemented with all amino acids except methionine and cysteine (27), and grown at 37 °C for 30 min. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 ml of cells to 1 mM and incubated at 37 °C for 15 min. During this incubation, IPTG would induce the expression of a sufficient amount of T7 RNA polymerase from the chromosome of BL21(DE3) cells; the T7 RNA polymerase initiates transcription of the KvAP cDNA from the T7 promoter of pET28a. Rifampicin (20 mg/ml in methanol) was then added to 200 μg/ml, and incubation was continued for an additional 45 min. Because rifampicin is an inhibitor of the E. coli RNA polymerase, expression of all chromosomal genes would be suppressed. Transcription of KvAP from the rifampicin-insensitive T7 RNA polymerase is unaffected, however. Cells were treated next with 10 μCi of [35S]methionine (MP Biomedicals) at room temperature for 5 min to allow selective radiolabeling of KvAP.

**Accessibility Studies of KvAP Cysteine Mutants—**Each cysteine mutant of KvAP was radiolabeled as above. Cells (1 ml) containing the radiolabeled mutant protein were pelleted, washed, and resuspended in 200 μl of PBSg (137 mM NaCl, 2.7 mM KCl, 10.0 mM NaH2PO4, 1.8 mM KH2PO4, and 5 mM glucose, pH 7.4). For the accessibility assay, cells were split into four equal aliquots: Aliquot i was treated with 5 mM N-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS; Molecular Probes), prepared in N,N-dimethylformamide, to modify extracellular cysteines. Aliquot ii was treated with 5 mM N-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS; Molecular Probes), prepared in N,N-dimethylformamide, to modify extracellular cysteines. Aliquot ii was treated with 5 mM AMS and 2% CHCl3 with immediate vortexing for 5 min to allow modification of intracellular cysteines. Aliquots iii (negative control) and iv (positive control) were left untreated. All four aliquots were incubated at room temperature for 30 min. Cells were pelleted, washed twice in PBSg, and solubilized in 30 μl of lysis buffer (2% (w/v) SDS, 6 μl urea, and 15 mM Tris, pH 7.4). Aliquots i, ii, and iv were incubated with 5 μM monomethoxy polyethylene glycol maleimide (mal-PEG, M, 5000; Nektar Therapeutics and Laysan Bio, Inc.) at 60 °C for 30 min. All aliquots were treated with an equal volume of 2× SDS loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 6 μl urea, and 125 mM Tris, pH 8.6) at 100 °C for 10 min and subjected to SDS-PAGE, followed by autoradiography.

**RESULTS AND DISCUSSION**

**Assay of Cysteine Accessibility of KvAP in Live E. coli—**MacKinnon and co-workers (28) reported overexpression of cloned KvAP in E. coli. Under the conditions of the experiment (rich culture medium), the expressed protein was detrimental to the growth of E. coli. Inclusion of Ba2++, a blocker of open potassium channels, in the culture medium reduced the toxicity, suggesting that the presence of excessive amounts of active KvAP could be toxic to the cell. In the present study, we have expressed KvAP in a minimal defined medium for a short period. Under these conditions, there appears to be no appreciable toxicity, as judged by the fact that KvAP expression could be readily
detected by pulse labeling (see below and Fig. 1); the apparent lack of toxicity may be attributed to low level expression of KvAP. This was fortuitous because this has allowed us to examine the topology of the channel in live E. coli.

To determine the membrane-aqueous boundaries of the VSD of KvAP, we have developed a simple and sensitive method that combined the in vivo selective radiolabeling of KvAP with site-directed cysteine modification. Fig. 1A shows how KvAP can be selectively radiolabeled in E. coli. In uninduced cells (lane 1), all cellular proteins were radiolabeled, and IPTG-induced cells (lane 2) showed an additional band corresponding to KvAP. In the IPTG- and rifampicin-treated cells (lane 3), where native protein synthesis is suppressed, only the KvAP band was detectable. Corresponding KvAP bands were absent in cells harboring the empty vector (lanes 5 and 6). Selective radiolabeling allowed easy detection of KvAP.

We expressed KvAP containing single cysteines as radiolabeled species and used AMS (Fig. 1B) (29, 30) to probe the position of introduced cysteines relative to the bilayer. AMS is a highly charged membrane-impermeable sulfhydryl reagent that reacts with the ionized thiolate (S−) group via its maleimide group. It can readily pass through the pores of the outer membrane of the cell and enter the periplasmic (extracellular) phase but cannot permeate the inner membrane. As such, when applied to intact E. coli, AMS would react with cysteines exposed to the periplasm. In the presence of 2% chloroform, however, AMS could cross the plasma membrane and also react with cysteines exposed to the cytoplasm. Although AMS could access cysteines present in the membrane phase while crossing, significant modification is not expected to occur because cysteines cannot ionize in the hydrophobic environment of the bilayer, and for maleimides to react cysteines must be in an ionized state. Modification of KvAP with AMS would increase the size of the protein by ~500 Da, but this size increase is too small to be detected by SDS-PAGE. For this reason, we have adopted the indirect gel-shift assay described by Lu and Deutsch (31). In this method, following modification with AMS cells are solubilized in SDS and then treated with a high molecular mass (~5000 Da) maleimide reagent, mal-PEG (Fig. 1B), to modify cysteines that have failed to react with AMS. This will result in a discernible gel shift on SDS-PAGE.

To validate the method, we examined the accessibility of three positions in the pore domain of KvAP (the structure of this region is uncontroversial), representing each of the environments: Ser178 (extracellular), Thr219 (membrane), and Ser280 (intracellular) (Fig. 1C). With all mutants, reaction with mal-PEG produced a higher molecular mass band (~33 kDa) representing PEGylated KvAP (Fig. 1C, last lane in each panel). However, in AMS-pretreated cells, gel shift was dependent upon the location of the cysteine: S178C failed to show any gel shift, indicating that Cys at position 178 was accessible to AMS; S280C showed gel shift only in the absence of chloroform, consistent with the intracellular location of Ser280. T219C showed gel shift under both conditions, consistent with the location of Thr219 in the membrane phase.

It may be pointed out that we did not observe complete PEGylation for any of the cysteine mutants, even when the reaction was performed under strong denaturing conditions (SDS/urea buffer). The reason for this is unclear, but this should not affect the interpretation of the results because we always compared the protective effects of AMS against a positive control run in parallel under identical conditions. The method is simple, robust, and highly sensitive, and, unlike other methods (16, 25), does not require overexpression and prior purification of the protein and can be adopted to determine the topology of any membrane protein that can be expressed in E. coli, even at low levels.

For the assay to be reliable, however, it is important that the protein is inserted into the inner membrane of the cell. Unlike...
the eukaryotic cells, *E. coli* lacks elaborate trafficking machinery, and expressed polytopic membrane proteins are directly incorporated into the inner membrane (32). However, if the expressed protein is misfolded, it would form inclusion bodies in the cytoplasm. Studies by MacKinnon and co-workers (28) showed that overexpressed KvAP could be extracted in milligram quantities with dodecyl maltoside and functionally reconstituted. Because inclusion bodies are insoluble in mild detergents such as dodecyl maltoside, this finding suggests that the majority of KvAP was inserted into the *E. coli* membrane. Under our experimental conditions, expression levels are even lower. Because low level expression is known to reduce inclusion bodies in the cytoplasm. Studies by MacKinnon and co-workers (28) showed that overexpressed KvAP could be extracted in milligram quantities with dodecyl maltoside and functionally reconstituted. Because inclusion bodies are insoluble in mild detergents such as dodecyl maltoside, this finding suggests that the majority of KvAP was inserted into the *E. coli* membrane. Under our experimental conditions, expression levels are even lower. Because low level expression is known to reduce inclusion body formation and promote membrane insertion (33), we expect that the vast majority, if not all, of expressed KvAP (and its mutants) was inserted into the inner membrane. We cannot completely rule out poor membrane insertion of some mutants, but the fraction of such mutants in our large screen is likely to be very low. Moreover, any anomalous accessibility resulting from such mutants could be identified because they would be expected to impact the scan pattern, for example by indicating an accessible residue in the middle of a perfect water-inaccessible transmembrane segment.

Membrane-aqueous Boundaries of S1 and S2—Having validated the method, we first examined the accessibility of residues starting from the N-terminal end of S1 to the C-terminal end of S2 using AMS alone (residues 21–85) and AMS with chloroform (residues 21–41 and 65–85) (Fig. 2 and supplemental Fig. 1). The data show that cysteines introduced at positions 30–47 in S1 and at positions 57–74 in S2 (Fig. 2, A and B, and summarized in C, far left) are inaccessible to AMS. Cysteines at positions 49 (S1) and 76–77 (S2) were inaccessible to AMS despite the accessibility of deeper residues, which could be because of an uneven membrane-aqueous border at these positions. These data highlight a stretch of 18 consecutive inaccessible residues in both S1 and S2, long enough to cross the plasma membrane. We do not know precisely how deep AMS could penetrate into the lipid, but given the small size of the maleimide group (2 Å) and its close proximity to the negatively charged sulfonate moiety (Fig. 1B), we do not expect this reagent to penetrate deep into the bilayer. Moreover, the phosphate lipid head groups may even restrict deeper entry of AMS into the bilayer through electrostatic repulsion with its sulfonate group.

FIGURE 2. Extra- and intracellular accessibility of cysteines at the membrane-aqueous borders of S1 and S2. A and B, gel-shift assay was performed on the indicated cysteine mutants as described for Fig. 1C using AMS alone (A) or AMS and chloroform (B); lanes T and C of each panel represent data from test (pretreated with AMS or AMS/chloroform) and control (no pretreatment) experiments, respectively; representative data from the same experiment and gel were edited together for clarity. The top band (indicated by arrowhead) on each gel corresponds to PEGylated KvAP. C, schematic topology of KvAP residues 21–85 (helices based on the crystal structure of the isolated VSD (Protein Data Bank code 1ORS)), summarizing our accessibility data (see supplemental Fig. 1 for a full scan), compared with previous studies. Black circles, accessible; white circles, inaccessible; gray circles, untested. For Shaker accessibility data KvAP equivalents are shown.
is known that the distribution of lipids in the native membrane bilayer, unlike the reconstituted systems, is asymmetrical and that the outer leaflet of the membrane represents a greater barrier to water movement than the inner leaflet (34). This may explain the greater accessibility of residues at the intracellular end to smaller water-soluble reagents.

Membrane-aqueous Boundaries of S3—When we examined the accessibility of the S3 segment, we found a total of 12 or 13 inaccessible residues, with the status of Pro95 undetermined because of nonexpression of P95C (Fig. 3, A and B). Not only is the size of the inaccessible region small, but the pattern is markedly different from that of S1 and S2: we see a stretch of 10 inaccessible residues (positions 94–103) followed at the extracellular end by both accessible (positions 104, 105, 107, 108, and 111; positions 104 and 105 are partially accessible) and inaccessible (positions 102, 103, 106, 109, and 110) residues. The small inaccessible region would be consistent with the crystal structures of KvAP, where S3 is divided into two short helices, and the separating nonhelical linker region extends a longer distance in the membrane compared with an α-helix (see Fig. 5B). Interestingly, the entire S3a helix was found to be accessible from inside, indicating the presence of a large water-filled cavity rather than a narrow crevice at the intracellular aspect of the VSD. This conclusion is consistent with the estimates from studies of the effect of low ionic strength on charge movement (35).

S3b showed variable extracellular accessibility but no intracellular accessibility. When mapped onto an α-helix, all accessible residues of S3b fall on one face of the helix (Fig. 3D). This suggests that S3b might be tilted with the accessible face toward the extracellular solution. It should be noted, however, that although in all crystal structures of KvAP (11) S3 is broken into S3a and S3b, in Kv1.2 it appears to be a single helix (10). If S3 were a single helix, and therefore compact, then S3 would be expected to span even shorter distance in the membrane, leaving much deeper water crevices at either end of S3. The accessibility of S3b is consistent with the EPR data on KvAP (25). With the Shaker channel, MTSET was reported to access much deeper positions (36, 37), but this could be attributed to the ability of MTSET to enter deeper into the bilayer (see above). These findings support the existence of an aqueous crevice lined by one face of S3b. In the avidin capture assay, which was designed to avoid residues in aqueous crevices, positions deeper than 109 were inaccessible (16).

Membrane-aqueous Boundaries of S4—S4 is the key component of the VSD, and its exposure to extracellular solvent dur-
The expression and activation of the KVAP channel has been confirmed by different technical approaches (16, 19, 38–41). Fig. 4A shows that at the extracellular end, a stretch of several consecutive S4 residues is accessible to AMS up to position 120. Because this position was reported to be accessible only in the activated state (19), it seems reasonable to assume that under the conditions of our experiment S4 is in its “up” state. In support of this, we see no accessibility for nine consecutive positions, 124–132, at the intracellular end of S4 for KVAP; the corresponding region in Shaker is inaccessible only in the up state (41). Thus, S4 appears to remain mostly in its up state during the course of incubation with AMS.

There are two accessible positions, 123 and 133, that fall within a stretch that is largely inaccessible. However, these results require cautious interpretation because these mutations most likely affect the structural integrity of the channel: Arg123 is engaged in an important salt bridge with a negatively charged residue in the VSD (9), and the equivalent of Arg133 in Shaker, Arg377, is critical for channel maturation and function (42).

At the intracellular end, accessibility is seen for residues 139–148. This region includes the C-terminal end of the S4–S5 helix and the linker that connects it to the bottom of S5. The border residues of the S4–S5 helix are not clearly defined in the KvAP crystal structures, but, based on a homology model built on the Kv1.2 structure, residues 135–143 are thought to comprise the S4–S5 linker (10). Five residues of this helix and following linker residues are accessible to AMS/chloroform.

Compared with the EPR study (25), we found more positions in the S4–S5 helix accessible to intracellular water. Another noticeable difference was the complete accessibility of residues (positions 144–148) linking the S4–S5 helix to S5, compared with one residue in the EPR study.

The S4 accessibility data suggest that the expressed channel is predominantly in its activated state. Consistent with this is the observation that heterologous expression of KVAP is toxic to E. coli and that the toxicity could be prevented with Ba2+/H11001, a blocker of Kv channels in their open state (28). The membrane potential of the inner membrane of E. coli is thought to be very negative, in the region of /H11002 100 mV. In lipid bilayer experiments, KVAP does not begin to activate until /H11002 60 mV. If the properties of KVAP in the native membrane were similar to those recorded from artificial bilayers (28), then one would expect the expressed channels to be closed in E. coli. The finding that this is not the case suggests that in the native environment KVAP is activated at a far more negative potential than in the artificial bilayers. Such an explanation would be consistent with the recent evidence that the voltage dependence of KVAP activation is greatly influenced by the lipid composition of the cell membrane (26).

CONCLUSION

By systematically examining the accessibility of each position, we have deduced the contour of the membrane-aqueous border of the VSD of KVAP in its activated conformation. The
results demonstrate that water-inaccessible regions of S1 and S2 correspond to the standard width of the membrane bilayer (∼28 Å), but those of S3 and S4 are considerably shorter (≥40%) (Fig. 5B). When the accessibility data are mapped onto the structure of the isolated VSD, the results reveal the location of aqueous crevices at both the extracellular and intracellular ends of the VSD, separated by a well defined membrane buried region (see supplemental Movie 1). It is likely that the crevices run much deeper into the interior of the VSD than revealed in our experiments because the size of our probe is larger than water. The high degree of water accessibility can explain the proton conducting properties of mutated VSDs of voltage-gated potassium (12) and sodium (14) channels and the Hv1 channel (7). Thus, the VSD of KvAP appears to be similar to that of eukaryotic K⁺ channels, where functional studies predicted such aqueous crevices. The current consensus is that these crevices reduce the thickness of the bilayer largely near the vicinity of S4. However, our data reveal that the bilayer compression is greater around S3 than S4. One face of S3b and the entire S3a were water-accessible, reducing the inaccessible region of S3 to just 10 residues. The structural basis for how S3 and S4 contribute to the formation of crevices is unclear, but S4 charges may play a primary role: in an attempt to form salt bridges with the lipid phosphates at the membrane-aqueous interface, the S4 arginines are thought to cause the bilayer to incurvate (to produce a biconcave effect) and produce hydrated crevices (11, 23, 24, 26). S4 has arginines in the outer and inner leaflets that may contribute to the formation of extracellular and intracellular water crevices, respectively. The S4-induced compression of the bilayer may cause S3 to adopt a conformation that would support the aqueous crevices. The structure of S3 appears to be well suited for this purpose because it is inherently flexible: positions of S3a, S3b, and the S2–S3 linker are different in different crystal structures of KvAP. Molecular dynamics simulations also support the inherent flexibility of S3, supporting the important role that it could play in voltage sensing (43).

The absence of crevices surrounding S1 and S2 indicates that they do not line the walls of the crevices and they probably do not move much during voltage sensing. On the other hand, the extensive exposure of S3 to water, together with the finding that S3a is the most dynamic region of the VSD (25), indicates that S3 helices likely undergo large movements in concert with S4 during gating. We propose that coordinated movements of S3 could modulate the configuration of crevices during gating, thereby reducing the energy barrier for translocation of a significant number of S4 gating charges (∼13 e) across the membrane electric field.

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