

Perspective

Voltage Sensor Movements

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The voltage dependence of K^+ , Na^+ , and Ca^{2+} channels is brought about by a voltage sensor that moves $\sim 12\text{--}13 e_0$ across the entire electric field (Schoppa et al., 1992; Hirschberg et al., 1996; Noceti et al., 1996). In the case of *Shaker* K^+ channel it is known which residues are responsible for this large amount of gating charge. This was found by measuring the total gating charge movement per channel after each of the putative charged residues (basic or acidic) were neutralized one by one (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). These studies revealed that the four most extracellularly located basic residues of the S4 segment (R362, R365, R368, and R371) and the most intracellular acidic residue in the S2 segment (E293) are the major contributors to the gating charge movement. The simple assumption that all these residues move across the entire electric field accounts for more than the measured total gating charge. This means that the movement of at least some of the charged residues is only partial within the electric field.

Conformational Changes

There is good evidence that conformational changes do occur in the region of S4 and S3 segments as a result of changing the voltage. By attaching fluorescent probes in these regions it has been possible to record changes in fluorescence produced by changes in membrane potential that can be attributed to modification of the quenching state of the fluorophore (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). Depending on the site, the time course and voltage dependence of the fluorescence changes may correlate loosely or strongly with the charge movement, indicating that the gating current is the result of conformational rearrangements in the channel molecule. However, these experiments do not give a direct indication of how much and how far the charged residues move. As the measured total charge per channel includes the product of the charge times the fraction of the field, the same charge movement can be satisfied by moving the charge in a static field or by changing the field in a static charge or a combination of both. Measurements of gating or ionic currents alone cannot resolve this question and a different type of measure-

ment is needed to decide the actual displacement of the charges.

Change in Exposure of Gating Charges Is State Dependent

A successful approach has been the probing of accessibility to residues from the extracellular or intracellular sides of the membrane as a function of membrane potential. The rationale of this method is that if voltage induces a change in position of the tested residue its accessibility may change when voltage induces a conformational change. The probes used have been cysteine reagents and protons. In the first case the residue under study is replaced by a cysteine and in the second case by a histidine. The results show indeed that the charged residues of the S4 segment change from internal exposure at hyperpolarized potentials to external exposure at depolarized potentials. The change in exposure of the charges is a direct indication that the basic residues reside in aqueous media in the resting and active conformations of the voltage sensor. The difference between probing with cysteine reagents as compared with protons is that with the latter all four basic residues change exposure while with the former the extreme positions get buried and do not cross all the way (Larsson et al., 1996; Yang et al., 1996; Yusaf et al., 1996; Baker et al., 1998; Starace et al., 1997; Starace and Bezanilla, 2001b). The difference is an indication that the basic residues reside in water-filled crevices that become very narrow toward the interior of the protein. The results of the effects of changing the ionic strength on the field sensed by the gating charges is also consistent with the idea of water crevices penetrating the protein core (Islas and Sigworth, 2001). The presence of water crevices starts delineating a possible mechanism of charge translocation during voltage sensing. Thus, a simple model for charge movement would be that a depolarization moves four charges from the internally connected water crevice to an externally connected water crevice and this would account for all the gating charge measured per channel. If the crevices were isopotential along their length the transfer of charge would be $16 e_0$, a value that exceeds the measured value of 12 to $13e_0$. However, as the crevices are expected to get narrow toward their end, there

should be a potential gradient along their length, therefore some charges would move less than the total span of the field and the total contribution could be reconciled to be $\sim 12\text{--}13 e_0$.

One consequence of the concept of water crevices penetrating the protein core is that the electric field may be more intense at the end of those crevices implying that all the charge movement can be achieved by a small conformational change. A proton channel is formed in hyperpolarized conditions when arginine 362 is replaced by histidine (Starace and Bezanilla, 1999) and at depolarized potentials when arginine 371 is replaced by histidine (Starace and Bezanilla, 2001b), indicating that in those regions the internal and external crevices meet each other through the single histidine residue that acts as a selectivity filter for proton conduction. These results also imply that the electric field is highly concentrated near residue 362 in the closed position and near residue 371 in the open position (see Fig. 13 in Starace and Bezanilla, 2001a) and that the length of the "gating pore" in these extreme positions spans a single charged residue because if there were more than one charged residue, protons would be excluded.

Recently, the introduction of site-directed electrochromic probes have made it possible to test the field strength in different sites of the Shaker K channel (Asamoah et al., 2002). The highest field strength was found to be near the second charged residue of the S4 segment and it was estimated to be almost five times stronger than in the bilayer. This result is consistent with the concept of water crevices penetrating the bilayer such that most of the electric field is concentrated in a very narrow region and in particular this result is in line with the results of histidine scanning.

Movements of the Sensor

There are several possible types of movement that can account for the basic experimental results of the total charge and its translocation. The most obvious movement would translocate the S4 segment perpendicular to the plane of the membrane from internal exposure to external exposure. Another possibility is a change in the tilt angle of the S4 that would change the exposure of the charged residues from inside to outside. Yet another possibility is a rotation of the segment to change the exposure of the charges. In all these basic movements it has been tacitly assumed that the S4 moves as a solid rigid body (and the actual movement could be any combination of these three modes of charge translocation). One proposal that is not based on the rigid body assumption hypothesizes that the segment may uncoil from its putative α -helical conformation to translocate the charges from inside to outside (Guy and Conti, 1990; Aggarwal and MacKinnon, 1996). Experi-

mental measurement of possible distance changes between residues of the voltage sensor during activation should give a direct answer to the question of the type of movement of the sensor.

Measurements of Intramolecular Distance Changes

Fluorescence resonance energy transfer (FRET) is a powerful technique that can resolve distances at atomic resolution and has been one of the methods employed to measure possible distance changes during activation of the Shaker K channel. Two variants of the technique have been used: FRET with regular organic fluorophores (Glauner et al., 1999) and LRET, which uses a lanthanide (such as Tb) as a donor molecule (Cha et al., 1999). In regular FRET the actual distance is difficult to estimate with precision because the orientation factor (κ^2 , Cantor and Schimmel, 1980; Selvin, 1996) is not known with precision, whereas with LRET fixing κ^2 to 2/3 gives measurements with a maximum of $\pm 10\%$ uncertainty because Tb emits isotropically. Since the labeling is done by attaching the fluorophores to cysteines, the tetrameric structure of the Shaker potassium channel offers the possibility of having access to four identical sites to be tested. Thus, the measured distances are between equivalent residues in different subunits forming the channel. In addition, all measurements have been limited to the extracellular side of the channel due to restrictions in the labeling protocols used in *Xenopus* oocytes, which is the expression system used in these experiments.

It is important to note that both techniques have demonstrated changes in distance of key residues in the neighborhood of the voltage sensor as a result of changing the membrane potential, but the actual values measured with the two techniques are different. An ingenious technique to measure distance based on a ruler that is attached to a cysteine on one end and contains a channel blocker at the other end (Blaustein et al., 2000) gave distances that were in very good agreement with the LRET results and differed significantly with the FRET results. In LRET it is possible to measure multiple distances because they appear as multiple time constants of the sensitized emission decay. As the measurements included distances between contiguous and across pore subunits it was possible to validate the technique by verifying that the two estimated distances were related as expected from geometry within 1 to 2 Å. In addition, the absolute distance measured between residues in the pore was validated with homologous residues from the crystal structure of KcsA (Doyle et al., 1998) within 1 Å.

There was an important common result from both techniques: the changes in distances observed with gating are quite small, not larger than $\sim 5\text{Å}$, which agrees with the idea that a large conformational change is not

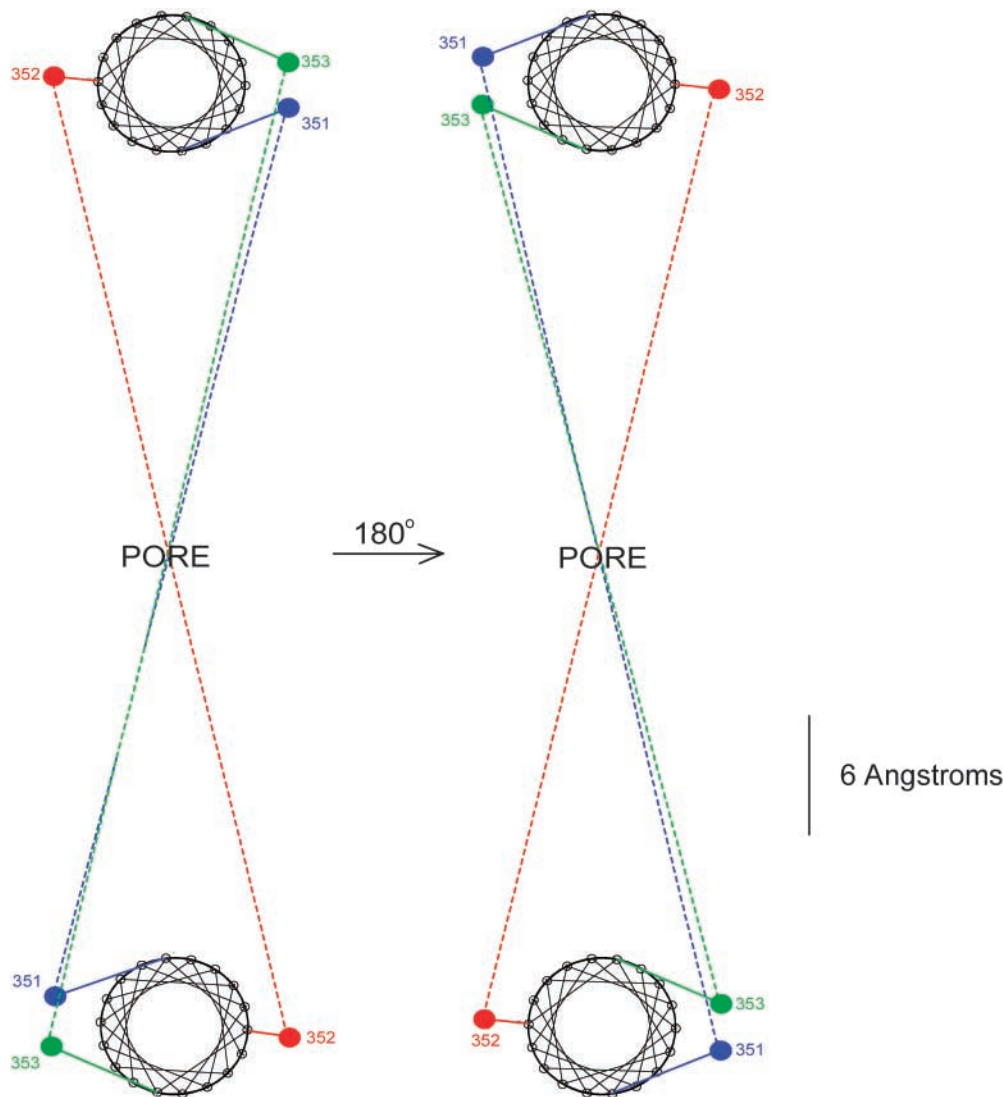


FIGURE 1. Schematic representation of one particular arrangement of the labels of three consecutive residues in an α helical wheel. The rotation has been set to 180° . For details see the text.

needed because the field is concentrated in a narrow region. In addition, the pattern of change of several amino acids suggested a rotation of the S4 segment. However, the actual changes measured differed in the direction for the same change in voltage. These differences could be reconciled by the uncertainties in both studies.

The technique of Cha et al. (1999) allowed the measurement of distance as a function of voltage in the same oocyte that gating current was measured and thus a direct correlation of the charge movement and the change in distance was obtained. In position 346, which is in the middle of the long loop joining the S3 and S4 segments, the change in distance amounted to ~ 3.2 Å. The interesting feature was that the voltage dependence of the distance change followed the same voltage dependence of the charge movement indicating that this conformational change was directly connected to the voltage sensor operation.

Rotation

In the study of Cha et al. (1999), a rotation of S4 was postulated because in three consecutive residues (351, 352, and 353) on the S3-S4 loop it was found that the intersubunit distance at the first residue increased, the second did not change and the third decreased when the membrane was depolarized. Although a 180° rotation was presented as a demonstration, it can be much less and still produce the same pattern of change in distances provided the second residue starts and ends at the same distance from the center of symmetry (the pore). The voltage-dependent distance changes of the first and third of the three consecutive residues are only ~ 1 Å, which is much smaller than an expected change from side chains in an α helix. However, this apparent discrepancy may be an indication of a different geometry of that portion of the S3-S4 linker. Let us consider as an example a 180° rotation. Assuming that typically side chains in an α helix are in a circle of 10 Å

diameter, one would expect that the total distance change could be as much as 20 Å, because the change of the two subunits would add. However, side chains, and in this case fluorophores, are not expected to be located in the same diameter of the α carbon and an extreme case is shown in the Fig. 1. As illustrated in the figure, a 180° rotation will produce a change of ~ 4 Å, still larger than the measured 1 Å. The next consideration is that these three amino acids are in fact in the linker between S3 and S4 segments and this linker at one point must be parallel to the plane of the membrane. The expected change in distance will decrease if the α helix becomes parallel to the membrane plane. Thus, in the case of expected 20 Å change it would become 1 Å if the angle formed with the membrane plane is 13°, or almost horizontal, and in the case of the expected 4 Å change (Fig. 1) it would become 1 Å if the angle with the membrane plane were 24°. These values are reasonable but cannot be taken too seriously because of so many uncertainties.

A different experimental approach also supports the rotation of the voltage sensor. Histidine scanning was initially done to study changes in exposure of the basic residues of the S4 sensor (Starace et al., 1997; Starace and Bezanilla, 2001b). However, when the scanning was extended to all other residues from 363 through 371 it was found that the voltage dependence of the charge movement was affected in a periodic fashion with respect to the position of the mutated residue. The $\Delta\Delta G$ computed from the change in voltage dependence of the charge as a function of the residue position showed a periodicity with an angle of 124° (Starace and Bezanilla, 2001a) with a periodicity index of 3.5. This angle is not too different from 120°, suggesting the possibility that the region that has the gating charge in the S4 segment may not be an α helix but a 3_{10} helix instead. However, if we keep the usual assumption that S4 is α helical there is an alternative explanation for this 120° periodicity, based on the rotation of the helix. The first four charges lay in a band that spans 180° and a vertical span of 10 residues times 1.5 Å/residue = 15 Å. As this band has all four gating charges that are known to be exposed, it corresponds to one of the aqueous crevices. For the residues that are just after the charges to fall into the same aqueous crevice we need a rotation of 120°. This is because a rotation of 100° will locate them in the same axial positions where the charges were but outside the crevice, because the charged residue and the next uncharged residue are actually displaced along the axis of the helix by 1.5 Å or 20° away from the crevice. The same reasoning applies to the next series of uncharged residues.

Change in S4 Tilt

There is no direct evidence for a change in the tilt of the S4 segment that may change the exposure of the

charged residues. LRET results (Cha et al., 1999) show a tilt in the S3-S4 linker that decreases on depolarization. If the S4 segment is aligned with the linker, S4 may also undergo a tilt change during activation.

Translation

The question whether the S4 moves perpendicular to the membrane was addressed only indirectly with the FRET and LRET experiments because those measurements were done across subunits on the outside of the membrane. Cha et al. (1999) used an indirect argument to argue that translational movement is not very large. For independently moving subunits the voltage dependence of the distance changes across subunits would not be expected to be monotonic because at intermediate potentials only one or two of the subunits would be displaced (Horn et al., 2000). Therefore the distance between the displaced subunit and the others would increase at small depolarizations but at larger depolarizations, when all four get displaced, the distance would decrease again producing a bell shaped curve of distance versus voltage curve. A 15 Å displacement of the S4 segment (moving all four basic residues out the membrane) would have been detected by the LRET measurements because the projection would be ~ 2.5 Å. The lack of such bell-shaped feature in the distance versus voltage curve indicates that the axial translation of S4 is significantly less than 15 Å.

A direct measurement of putative distance changes across the membrane is needed but it has been difficult to label channel sites on the inside of the oocyte membrane. Recently, Starace et al. (2002) presented a technique that allows estimation of distances from the inside to the outside of the channel protein based on the use of an inserted enhanced green fluorescence protein (eGFP). The eGFP coding sequence was inserted ~ 20 amino acids away from the COOH terminus of the S6 and was used as a donor molecule for FRET studies to acceptor fluorophores conjugated to site-directed cysteines on the outside of the protein. The acceptor molecule was an MTS derivative of tetramethylrhodamine (MTSR) that can be bound to cysteine and cleaved off by DTT. In the transmembrane FRET experiments, MTSR was attached to a specific external cysteine and the fluorescence of the eGFP donor in the presence of acceptor (F_{DA}) was measured for a range of potentials. The acceptor was then cleaved off by DTT and the donor fluorescence in absence of acceptor (F_D) was measured for the same range of potentials. From these two measurements the energy transfer was computed as $E = (1 - F_{DA}/F_D)$ and from this an estimate of distance was obtained using a κ^2 of 2/3 in the computed R_0 (the distance that transfer 50% of the energy). The results from several acceptor sites spanning both S1-S2 and S3-S4 linkers indicated a decrease of trans-

membrane distances as the sites were closer to the extracellular beginning of each segment, as expected. However, the change in distance with voltage did not exceed 2 Å in any of the sites regardless of whether they were close or far from the S4 segment. In fact, in many cases the transmembrane distance decreased rather than increased with depolarization. The possibility that the changes seen are the result of a combined movement of the S4 segment in one direction and the eGFP in the opposite direction is unlikely because the observed distance changes in sites in the S1-S2 loop, which are far from S4, are not different from the sites in S3-S4 loop which are close to S4. The resolution in these experiments is appropriate because, given the distance between donor and acceptor (~ 75 Å), there is $\sim 13\%$ energy transfer and an easily detectable change of 1% in energy transfer corresponds to ~ 1.1 Å change in distance, certainly more than enough to detect a 10–15 Å of axial translation as proposed in some S4 models.

Is it possible that the small transmembrane distance changes are the result of a significant tilt of the S4 segment with respect to the perpendicular to the plane of the membrane? We can take two extreme cases for the position of the eGFP, which is unknown, to compute the projection of a 15 Å displacement of the S4 segment. In the first case the line joining the sites in the S3-S4 linker and the eGFP is perpendicular to the plane of the membrane. In this case, if the S4 segment moves exactly perpendicular to the membrane, the expected measured change should be 15 Å. If the S4 is tilted by as much as 45° then the expected measured change should be as large as 11 Å. In the second case, we consider that eGFP is located near the center of symmetry of the molecule (in the plane of membrane ~ 40 Å away from the position of S4). In this case, if the S4 segment moves exactly perpendicular to the membrane, the expected measured change is 13 Å. If the S4 is tilted by 45° , then the expected measured change should be as large as 5 Å. These estimates indicate that a large translation, such as the expected 15 Å, should have produced a large detectable change in this FRET technique. The absolute distances measured are uncertain in FRET measurements because the orientation factor is not known, but the distance changes have much less uncertainty than the absolute distances.

So far we have been assuming that the S4 behaves like a rigid body carrying all the charges simultaneously. If the segment uncoils during activation and the break occurs in the segment and not in the linker, the transmembrane FRET results may not reveal the distance change occurring inside the segment and still a translation might be possible. This possibility can be tested by extending the transmembrane FRET measurements to acceptor sites well within the S4 segment and such experiments are now in progress.

In Shaker the linker between S3 and S4 is ~ 31 residues, allowing a very flexible stretch for a possible translation of S4 out of the membrane plane. However, the experiments of Gonzalez et al. (2001) have shown that this linker can be shortened and even eliminated and the channel still opens. Thus, a channel with a linker of only two residues still opens with the same maximum open probability and moves all the gating charge. If the S4 segment moves out of the plane of the membrane a total of 15 Å this would not be possible unless the S3 segment is also dragged with it. In this context, it is interesting to note that the recently cloned bacterial Na channel (NaChBac; Ren et al., 2001) has only two residues in the S3-S4 linker.

The results of Gonzalez et al. (2001) showed a periodicity in the midpoint of the activation curve or the time constant of activation as a function of the number of residues left in the linker. This periodicity was 100° , indicating an α helical structure in the COOH terminus side of the linker. If the S4 is also α helical this would indicate that the same structure is maintained in the linker as far as it can be measured with this technique which is ~ 6 residues from the NH_2 terminus of S4. This estimate of α helical structure is only three residues away from the three consecutive residues that Cha et al. (1999) found to follow a possible rotation.

Models of Voltage Sensor Movement

The available evidence does not allow the formulation of a definite model of voltage sensor movement mainly because it is incomplete. However, proposals can be made that certainly will require improvements or modifications as more measurements or, even better, a crystal structure becomes available. Cha et al. (1999) and Bezanilla (2000) proposed a simple rotational model that would expose, alternatively, the first four basic residues to an internally connected crevice at hyperpolarized potentials and to an externally connected crevice at depolarized potentials.¹ Just a simple rotation cannot account for all the data because when all the charges are in one crevice, residues on the back side of the helix would be accessible to the other crevice and should have an exact opposite voltage dependence. This pattern is not exactly followed experimentally. Therefore, some side chain rearrangement or translational or tilt change of S4 or movement of the adjacent segments may also occur. In fact, we do not even know for sure whether the S4 segment is α helical or perhaps a 3_{10} helix (see above) or whether there is a break in the structure and the movement of the charge involves uncoiling and recoiling of the S4 helix.

¹The simple rotational model including the simulation of single gating shots, single channels, gating currents, and ionic currents is available at: <http://pb010.anes.ucla.edu/model/rotmodel.html>.

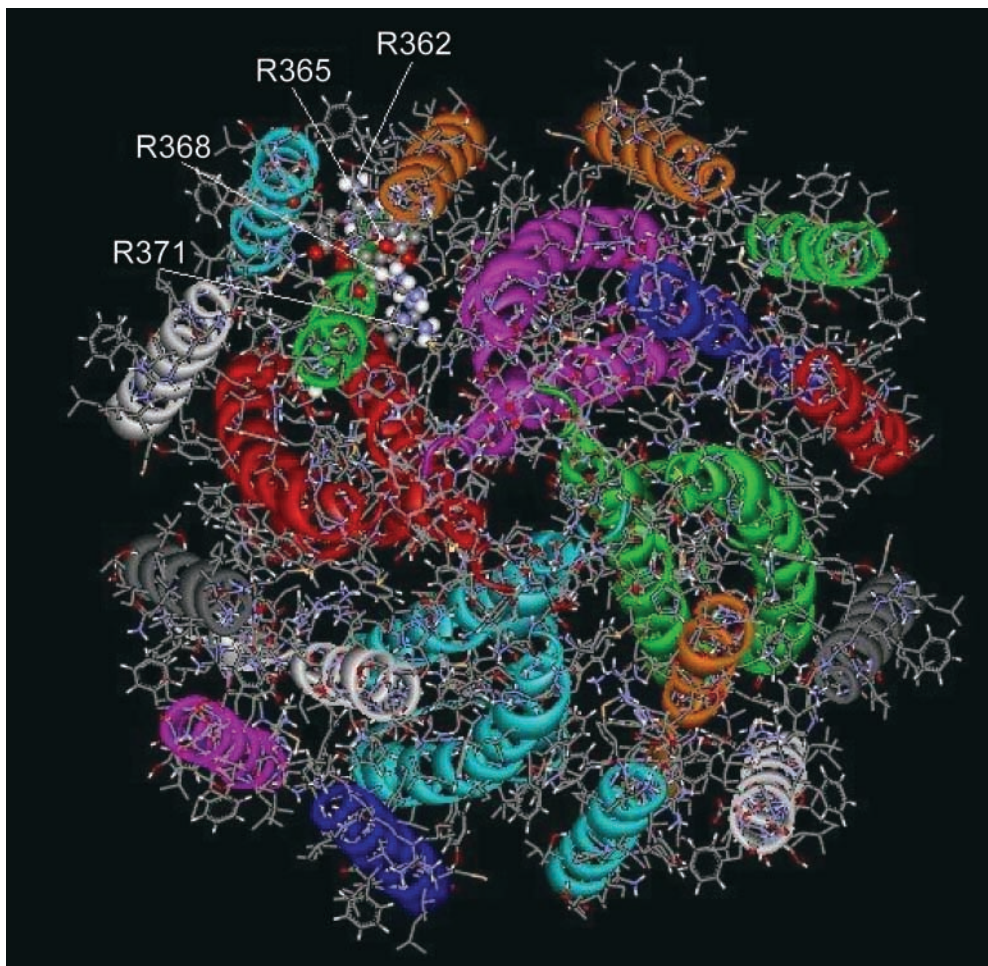


FIGURE 2. Intracellular view of the minimized structure in the closed state. The sequence of segments in the left upper quadrant is gray, S1; blue, S2; orange, S3; green, S4; red, S5 pore loop and S6. The emphasized side chains shown in the upper quadrant correspond to the basic residues showing that they are all in the intracellular-facing side of the S4 segment.

With all these caveats, a model of S4 movement should be taken as tentative. In this section, instead of illustrating the sensor movement with just another cartoon we have used a molecular structure, with the atomic constraints of molecular modeling. The basic three-dimensional topology is taken from the work of Roux (2002) who has produced a series of likely three-dimensional Shaker K models by minimizing the structure using available experimental constraints. These constraints have been the measured distances (Cha et al., 1999; Blaustein et al., 2000), the electrostatic interactions (Papazian et al., 1995; Tiwari-Woodruff et al., 2000) and the systematic scanning of residues to test exposure (Monks et al., 1999; Hong and Miller, 2000; Li-Smerin et al., 2000; Li-Smerin and Swartz, 2001). Roux found four types of structures that were equally likely but the recent results of the Papazian laboratory (Silverman, 2002) with the *eag* channel makes one of the structures much more likely. The minimized structure by Roux is in only one conformation and satisfies all the above constraints simultaneously regardless whether they would apply to the closed or open states.

Taking Roux's structure as a starting point, I attempted to satisfy two possible basic structures: one in the closed state and another in the open state where, for simplicity, the only conformational change allowed was a rotation of the S4 segment. (Though the model depicts an attempt at molecular reality, I wish to emphasize that it should be viewed with the same skepticism as should the more conventional representations of channel gating.) The procedure was a manual rotation of the S4 segment with redistribution of side chains to eliminate clashes. This initial procedure was followed by energy minimization with the Gromos96 force field as implemented by the Swiss SPDB program. The result is shown in Fig. 2 for the closed state as viewed from the intracellular side. The tilt of the S4 segment was 22° , therefore the S4 helix serves as a barrier that separates the outside from the inside. In the closed state the basic residues can be seen from an inside view of the channel (Fig. 2). To facilitate the view of both conformations, Fig. 3 shows an extracellular view after the molecule has been tilted 22° such that the S4 segment becomes perpendicular to the plane of the figure. It can be observed

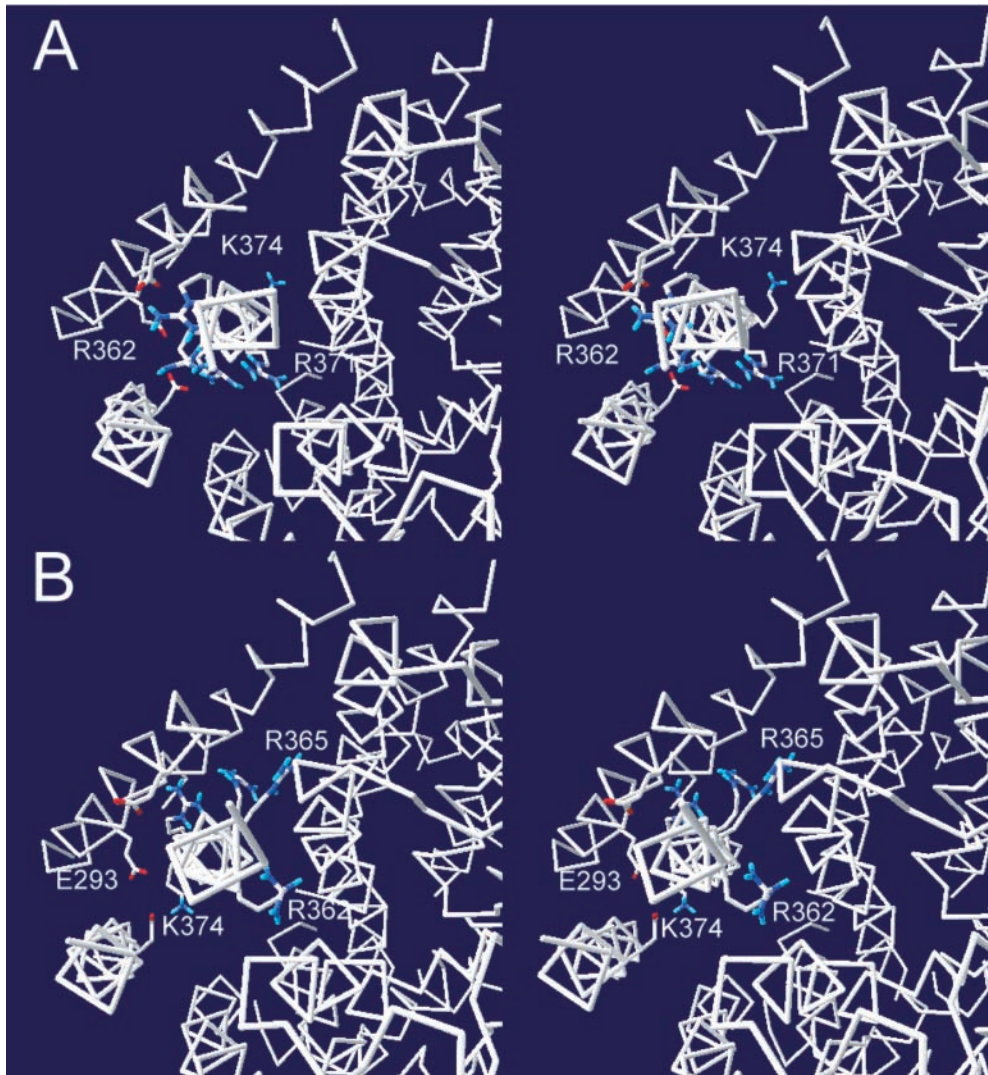


FIGURE 3. Stereo extracellular view of the voltage-sensing region of one quadrant of the minimized model. The molecule has been tilted to position the S4 segment perpendicular to the plane of the figure ($\sim 22^\circ$). Notice that in the closed state (A) the basic residues point toward the bottom left corner of the figure that corresponds to the intracellular side, while in the open state (B) they rotate $\sim 180^\circ$. Some residues are labeled for orientation. Residue E293 is in the S2 segment and D316 is in the S3 segment. Due to the tilt of the S1 and S2 segments they appear to be aligned.

that in the closed state the first four basic residues of S4 point in the inward direction: this would correspond to the inner crevice. In the open state the rotation of the S4 has made all four basic residues to point in the outward direction: this would correspond to the external crevice. Residue K374 becomes close to residues D316 of S3 and E293 of S2 in the open state in accordance to the electrostatic interaction of Papazian et al. (1995). Residue E283 is relatively close to R368 and R371 but a rotation of S2 may increase this proximity even more. A rotation of S2 may also change the position of E293, possibly across the electric field, which would be in agreement with the result of Seoh et al. (1996) that showed this residue to be part of the gating charge. This rotation has not been implemented here. Nor has there been an attempt in this modeling to couple the rotation of the S4 segment with channel opening that probably requires a large conformational change in the S6 segment (Perozo et al., 1999; Jiang et al., 2002).

The energy minimization located the side chains of the basic residues in a pattern that can account for water crevices in the closed and open configurations. It is interesting to note that the inside crevice has a large vestibule in the closed state (Islas and Sigworth, 2001). It is possible that during the opening step, the removal of the long and bulky side chains of the basic residues would increase the available volume in the vestibule. This, in turn, would facilitate the rotation and bend of the S6 segment into that volume while the pore enlarges and the channel becomes conductive. In this model there is not an equivalent vestibule in the external crevice and the space seems a bit more restricted there. It is then possible that the opening step requires, in addition to the rotation, a small translation, a tilt change or a rearrangement of the contiguous segments that would increase the exposure to the outside of the basic residues in the open state. We also learned from this modeling that the minimization made significant rearrangements in side chain positions that should

have consequences in accessibility of other residues in addition to the charged groups.

Figs. 2 and 3 indicate that a rotation is possible for this specific molecular model of Shaker. It is expected that as the number of experimental distance measurements increase and other side chain proximity contacts are discovered across segments, the modeling will become constrained. A great opportunity to study the movement of the voltage sensor has been presented by the cloning of NaChBac (Ren et al., 2001), a bacterial voltage-dependent sodium channel that can be purified in mg quantities. Using this protein we have obtained direct distance measurements using LRET (unpublished data). It is also likely that a crystal structure of NaChBac may become available in the not too distant future. What appears certain is that this model will require many improvements and modifications, and perhaps a very different model will emerge that may combine many of the possible movements of the S4 residues and neighboring segments to achieve the charge transfer of the voltage sensor.

Conclusions

The translocation of $13 e_0$ across the electric field is responsible for the voltage dependence of Na^+ , K^+ , and Ca^{2+} channels. Upon depolarization the basic residues are relocated from internal exposure in a water crevice to an external water crevice. Most of the electric field is concentrated in a narrow (changing) region of the protein so that the charge transfer is mediated by small conformational changes that may involve rotation, change in tilt and a minimal translation of the S4 segment with possible simultaneous rearrangements of the S2 and S3 segments.

I am indebted to Dr. B. Roux who provided the coordinates of the structure used as the starting point for the model shown in Figs. 2 and 3. Many thanks to Drs. Dorine Starace and Baron Chanda for reading and commenting on the manuscript.

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