S4–S5 linker movement during activation and inactivation in voltage-gated K⁺ channels

Tanja Kalstrup* and Rikard Blunck*✉,1

*Department of Pharmacology and Physiology, Université de Montréal, Montréal, QC H3C 3J7, Canada; and ✉Department of Physics, Université de Montréal, Montréal, QC H3C 3J7, Canada

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The S4–S5 linker physically links voltage sensor and pore domain in voltage-gated ion channels and is essential for electromechanical coupling between both domains. Little dynamic information is available on the movement of the cytosolic S4–S5 linker due to lack of a direct electrical or optical readout. To understand the movements of the gating machinery during activation and inactivation, we incorporated fluorescent unnatural amino acids at four positions along the linker of the Shaker Kᵥ channel. Using two-color voltage-clamp fluorometry, we compared S4–S5 linker movements with charge displacement, S4 movement, and pore opening. We found that the proximal S4–S5 linker moves with the S4 helix throughout the gating process, whereas the distal portion undergoes a separate motion related to late gating transitions. Both pore and S4–S5 linker undergo rearrangements during C-type inactivation in presence of accelerated C-type inactivation, the energetic coupling between movement of the distal S4–S5 linker and pore opening disappears.

Voltage-gated potassium channels | unnatural amino acids | voltage-clamp fluorometry | inactivation | Anap

Voltage-gated potassium (Kᵥ) channels are responsible for repolarization of the membrane potential during neuronal and cardiac action potentials and regulate neuronal excitability among many other physiological processes. Mutations in Kᵥ channel genes thus result in severe neuronal and cardiac diseases and channelopathies, such as episodic ataxia, epilepsy, and cardiac arrhythmia. Kᵥ channels thus constitute therapeutic drug targets for treatments of diverse diseases (1). Kᵥ channels’ central role in heart and brain underscores the importance of understanding their mechanisms in detail.

All mammalian Shaker-like Kᵥ channels (Kᵥ1 channel subfamily) share the characteristic assembly of four subunits which each consists of six transmembrane helices containing a voltage sensor (S1–S4) and a pore region (S5–S6; Fig. 1A) (2, 3). The voltage sensor (VS) contains positively charged arginine residues in the S4 helix, which are responsible for the generation of gating currents as they rearrange according to changes in the electric field they sense across the membrane (4). This conformational change results in pore opening through a mechanism called electromechanical coupling (5–12).

Although dynamic information is lacking and the underlying molecular driving forces are not fully understood, a consensus model of the coupling process has emerged (13, 14). First, initial S4 charge movements (Q1) occur independently in each VS upon membrane depolarization, applying a force onto the S4–S5 linker as they move upward within the membrane. Then, during a second S4 charge movement (Q2), energy is released to the pore domain in a cooperative conformational change, which finally results in widening of the bundle crossing at the intracellular S6 gates. The opening step has been shown to occur cooperatively among the subunits (15, 16) and comprises at least two transitions (17). In addition to being covalently bound to the pore domain via S5, the S4–S5 linker interacts directly with the S6 bundle crossing via noncovalent interactions of both the same (6, 7, 11, 18) and the neighboring subunit (19). Because dynamics of the S4 helices and the inner S6 gate are associated with gating currents and ionic currents, respectively, the roles of each region in the activation process have been well established. The S4–S5 linker movement, on the contrary, is intrinsically not associated to any charge movement. Moreover, the intracellular position of the S4–S5 linker has made it inaccessible for fluorophore-labeling. Consequently, only limited dynamical information is available for the S4–S5 linker movement (20).

To investigate S4–S5 linker dynamics, we have made use of the stop codon suppression technique for the genetic incorporation of a fluorescent unnatural amino acid (Anap) (17, 21, 22), enabling specific labeling of intracellular sites for the use of voltage clamp fluorometry (VCF) in proteins expressed in _Xenopus_ oocytes (17, 23). We aimed to address questions as to where the transition between the independent voltage sensor movement and the cooperative pore opening occurred and whether the S4–S5 linker rearranges as a rigid body throughout the gating process or with a degree of flexibility, allowing conservation of the energy provided by the voltage sensor. To this end, Anap was inserted into the S4–S5 linker of the Shaker Kᵥ channel. By labeling the external part of the S4 helix with a thiol-reactive dye, we were able to compare movements of the voltage sensor and of the S4–S5 linker simultaneously.

**Results**

Assessment of Functional Expression with Anap. All 12 positions along the Shaker S4–S5 linker (residues 381–392) were scanned for Anap insertion (Fig. 1B). Proper protein maturation and folding were determined from the presence of both ionic and thiol-reactive labeling (Fig. 1C).

**Significance**

Excitability in heart and nervous system is based on sensing and propagation of the membrane potential by voltage-gated ion channels. Despite the increasing availability of high-resolution structures of voltage-gated ion channels, key questions about their dynamics remain elusive. Here we followed the movements of the gating machinery on the cytosolic surface of the Shaker Kᵥ channel by introducing a fluorescent non-canonical amino acid at different positions along the linker between voltage sensor and pore. We can thus map the movement of each position and reconstruct the dynamics of the gating machinery during activation and inactivation. Considering that the Shaker channel serves as a model system, the mechanisms are likely conserved among those Kᵥ channels containing a sizeable S4–S5 linker.

Author contributions: R.B. designed research; T.K. performed research; T.K. and R.B. analyzed data; and T.K. and R.B. wrote the paper.

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1To whom correspondence should be addressed. Email: rikard.blunck@umontreal.ca.

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gating currents. Four positions resulted in robust expression and voltage-dependent fluorescence changes with Anap inserted at L382, R387, K390, and A391, whereas four did not express (G381, Y385, T388, and G392). The four remaining residues either showed limited expression (Q383 and L389) or defective channel opening (I384 and G386). This agrees with the essential role that I384 has been shown previously to play for electromechanical coupling (8). When mapping the other residues onto the available Kv channel crystal structures (2, 3), the nonexpressing residues face inward toward the center of the channel (black in Fig. 1B), whereas expressing residues point outward, away from the channel (red in Fig. 1B). This finding agrees with the fact that a large amino acid like Anap is more likely to sterically disrupt protein folding at positions that interact with the protein versus positions that point outward into the cytosol or into the lipid bilayer.

It was recently shown that Shaker channels lacking the pore domain express as functional isolated voltage sensor domains (iVSD), giving rise to gating pore currents (24). These currents were also observed in mutants with a stop-codon insertion in the S4–S5 linker in the absence of Anap or pAnap (SI Appendix, Fig. S1 A and B). However, although iVSD currents also occurred when Anap and pAnap were present, their amplitude depended on the success of Anap incorporation, such that iVSD current amplitudes were considerably smaller in oocytes expressing full-length channels (4.4 μA ± 6.7 at −170 mV) compared with oocytes in which full-length channels were absent (20.4 μA ± 7.9 at −170 mV; SI Appendix, Fig. S1C). Having ensured that iVSD currents were not altering gating kinetics and did not form heterotramers (SI Appendix), we were able to proceed to the analysis of the fluorescence signals.

**The C Terminus of the S4–S5 Linker Rearranges After S4 Activation.** The four S4–S5 linker mutants L382Anap, R387Anap, K390Anap, and A391Anap of full-length Shaker exhibited robust expression levels, with Anap changes showing relative fluorescence intensity changes (ΔF/F) in the range of 0.2–4% in response to depolarization (Fig. 2A). Because fluorescent labeling either by insertion of Anap or the thiol-reactive fluorophore tetramethylrhodamine-maleimide (TMR) might influence kinetics and energy of voltage sensor movement and pore opening, we can only directly compare voltage sensor movement, movement of the S4–S5 linker, and pore opening in the same mutant, i.e., tracking all parameters simultaneously. We showed previously that fluorescence originating from TMR attached to the extracellular end of S4 reliably follows gating charge movement (8). Therefore, the stop codons were inserted in the Shaker-A359C background, to compare S4–S5 linker movement (Anap signals) with voltage sensor movement (A359C-TMR signals), simultaneously (Fig. 2A). In addition, the ionic current—reporting pore opening—was measured at the same time (Fig. 2A).

As expected, the voltage dependence of voltage sensor movement (VF-TMR) for all four mutants developed at less depolarized potentials compared with pore opening [conductance voltage relation, GV (Fig. 2 B–E); ΔV_1/2 L382Anap −67 mV; R387Anap −33.6 mV; K390Anap −27.3 mV; A391Anap −43.2 mV]. In contrast, comparison of the voltage dependencies of voltage sensor movement (VF-TMR) and movement at the S4–S5 linker (VF-Anap) varied with the monitored position in the S4–S5 linker. Although the signals overlapped in L382Anap (Fig. 2B), the movements at R387, K390, and A391, in the S4–S5 linker, were shifted to more depolarized potentials compared with voltage sensor movement by 29, 21, and 21 mV, respectively (Fig. 2 C–E and Table 1). However, movement at all three positions was still occurring at less depolarized potentials than pore opening (GV; ΔV_1/2 R387Anap −14.9 mV; K390Anap −6.6 mV; A391Anap −21.4 mV).

To ensure that the signal was caused by conformational changes at the labeling position, we excluded that the fluorescence was influenced by potassium flux through the nearby pore entrance. Conductance might alter local ion concentrations and, thereby, the local electric field, which could influence fluorescence signals. To obtain good fluorescence signals, we need to express the conducting channels at high levels. The accumulation of external K⁺ at high expression levels also led to slowing of channel deactivation as evidenced by the slower tail currents (Fig. 2A) (25, 26). We used Ba²⁺ to block the ionic current and to give a signal similar to K⁺ and can enter the pore but blocks conductance once it reaches the selectivity filter with minimal effects on voltage sensor activation (27). Despite significant block by external Ba²⁺ in R387Anap and K390Anap channels, the total Anap fluorescence change was not diminished, and the fluorescence time course and voltage dependency remained unaffected, indicating that the fluorescence signals were indeed caused by conformational changes at the labeling site (Fig. 2 F and G). The results suggest that only the N-terminal part of the linker (L382) moves together with the S4 helix during activation, whereas the more distal part of the linker rearranges later in the activation process.

**The S4–S5 Linker Experience C-Type Inactivation-Related Rearrangements.** A second fluorescence component of opposite direction appeared in A391Anap at depolarizations more positive than 60 mV (Fig. 2E). An overlap of the current and fluorescence time course during a prolonged depolarization pulse revealed that the second fluorescence component correlated kinetically with C-type inactivation (Fig. 2F). Moreover, the second fluorescence component was sensitive to application of 4-aminopyridine (4-AP; Fig. 2H). 4-AP is a Kv channel blocker, which prevents Kv channels from entering the final concerted pore opening transition and thus also C-type inactivation (28).

This was confirmed in nonconducting W434F mutants, in which ionic currents are blocked due to accelerated C-type inactivation (29). In agreement with the accelerated C-type inactivation (29), the second Anap fluorescence component in A391Anap-W434F channels appeared faster and larger than in absence of the W434F mutation (Fig. 3A and E). The FV from A391Anap-W434F channels could no longer be fitted to a single Boltzmann relation because the onset for C-type inactivation now occurred at lower potentials than channel opening did (Fig. 3E). These findings indicate that A391Anap probes rearrangements that are associated with C-type inactivation, in addition to those associated to activation.

A second smaller Anap component of opposite direction also appeared in K390Anap-W434F channels (Fig. 3D). For both K390Anap-W434F and A391Anap-W434F mutants, the second fluorescence component was 4-AP sensitive as demonstrated in the fluorescence time course and in the FV obtained before and after 4-AP application (Fig. 3 F–I). Because the second fluorescence component in the conducting K390Anap channels is
absent or too small to be measured, it was not possible to verify whether the kinetics of C-type inactivation correlated with the fluorescence signal as was the case with A391Anap (Fig. 2H).

The W434F data, nonetheless, suggest that Anap is influenced by C-type inactivation at position K390 in the same way as at position A391.

C-Type Inactivation Allows the S4–S5 Linker to Assume Final Position After Voltage Sensor Activation. The most remarkable difference in the W434F background was that the shift between voltage sensor (TMR) and S4–S5 linker (Anap) movement was strongly diminished (Fig. 3B–E). The disappearance of the shift indicates that in presence of W434F, the entire S4–S5 linker undergoes its conformational change upon voltage sensor activation. In the conducting channel, in contrast, the C terminus of the S4–S5 linker cannot enter its final position yet after voltage sensor activation but is energetically coupled to an additional transition.

The question remains whether also the sequence of events has altered, i.e., voltage sensor movement, followed by linker movement and finally pore opening. To answer this, we considered

**Table 1.** \( V_{1/2} \) and \( z \) values were obtained from single Boltzmann fits of FV and GV curves of the indicated mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>TMR FV ( V_{1/2} ), mV</th>
<th>TMR FV ( z )</th>
<th>Anap FV ( V_{1/2} ), mV</th>
<th>Anap FV ( z )</th>
<th>GV ( V_{1/2} ), mV</th>
<th>GV ( z )</th>
</tr>
</thead>
<tbody>
<tr>
<td>L382Anap</td>
<td>(-39.2 \pm 2.7)</td>
<td>1.08 ( \pm 0.09)</td>
<td>(-43.9 \pm 0.7)</td>
<td>1.91 ( \pm 0.11)</td>
<td>27.8 ( \pm 1.0)</td>
<td>1.23 ( \pm 0.05)</td>
</tr>
<tr>
<td>R387Anap</td>
<td>(-27.4 \pm 2.1)</td>
<td>1.27 ( \pm 0.10)</td>
<td>(1.3 \pm 1.3)</td>
<td>1.46 ( \pm 0.16)</td>
<td>16.2 ( \pm 0.5)</td>
<td>1.94 ( \pm 0.07)</td>
</tr>
<tr>
<td>K390Anap</td>
<td>(-42.9 \pm 1.9)</td>
<td>1.26 ( \pm 0.09)</td>
<td>(-22.2 \pm 0.9)</td>
<td>1.24 ( \pm 0.09)</td>
<td>(-15.6 \pm 0.7)</td>
<td>1.74 ( \pm 0.06)</td>
</tr>
<tr>
<td>A391Anap</td>
<td>(-46.4 \pm 1.3)</td>
<td>1.37 ( \pm 0.08)</td>
<td>(-24.6 \pm 1.0)</td>
<td>2.07 ( \pm 0.20)</td>
<td>(-3.2 \pm 1.0)</td>
<td>1.47 ( \pm 0.61)</td>
</tr>
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Fig. 2. Fluorescence profile of conducting channels. (A) VCF recordings of (Upper) ionic currents, (Middle) Anap, and (Lower) TMR (green) fluorescence changes obtained from oocytes expressing each of the S4–S5 linker mutants. The arrow next to each fluorescence signal represents 1% \( \Delta F/F \). Anap FV, TMR FV, and GV curves of (B) L382Anap, (C) R387Anap, (D) K390Anap, and (E) A391Anap channels. Each dataset is fitted to a Boltzmann distribution (Methods). Error bars indicate mean \( \pm \) SD. (F and G) Current and fluorescence output upon depolarization from \(-90\) to \(20\) mV before and 30 min after external application of 40 mM barium in (F) R387Anap and (G) K390Anap channels. (Inset) Anap FV obtained before and after block. (H) Anap fluorescence (black) and ionic currents (red) of A391Anap channels upon prolonged 1-s depolarization from \(-90\) mV to (Left) 50 and (Right) 100 mV. In gray, ionic current and Anap fluorescence after application of 5 mM external 4-AP for the 50-mV pulse is shown.
the dynamic information. The time constants obtained from exponential fits of the fluorescence and gating currents were plotted as a function of the test pulse (Fig. 4A–D). The mutants exhibited similar patterns for TMR and gating currents, each with two components: one in the 1- to 10-ms range and one in the 10- to 100-ms range (green and black symbols, Fig. 4A–D) except for A391Anap-W434F gating currents, which only could be fitted to one exponential (Fig. 4D). This could be either because the two components are kinetically similar (yet have different voltage dependencies because the QV was fitted to a double Boltzmann relation) and therefore appear indistinguishable or because the slower component is too small or too slow to be detected during the test pulse. In fact, A391Anap-W434F generally expressed less than the other linker mutants yielding maximally 1 μA of gating currents when depolarizing from −90 to 50 mV. It is therefore possible that the slow component in the gating currents disappeared within the experimental noise. Nevertheless, these findings suggest that the kinetics of the upper S4 movement and the charge displacement were not markedly affected by the relative position of Anap in the S4–S5 linker in the W434F mutant.

However, how did the voltage sensor kinetics compare with the linker movement monitored by Anap? In L382Anap-W434F, two Anap components were observed: one in the 1- to 10-ms range and another in the 10- to 100-ms range (red symbols, Fig. 4A). Although the fast TMR component is 1–5 ms slower than the fast Anap component, the kinetics of TMR, Anap, and gating currents are comparable. The result agrees with the overlapping FVs and QV for L382Anap-W434F (Fig. 3B). When overlapping the fluorescence time courses and charge displacement, it is seen that TMR relaxes slower than Anap and that Anap is kinetically closer to the charge displacement in the first milliseconds when...
the fast component is dominant (arrow, Fig. 4E). Furthermore, the absence of a delay in the onset of the Anap fluorescence compared with TMR indicates that their movements are prompted simultaneously (black arrow, Fig. 5A). These data are similar to earlier data of V234Anap on the S1 helix, which is only 9.6 Å away (17) (Fig. 4F). Based on the kinetic analysis and FV and QV comparisons, the results show that the L382 movement is similar to that of the voltage sensor.

For the C-terminal part of the S4–S5 linker, however, Anap fluorescence only exhibited a single time constant (red circles, Fig. 4B–D) when disregarding the 4-AP-sensitive slow component of opposite direction in K390Anap-W434F and A391Anap-W434F (red open squares, Fig. 4C and D). In contrast to L382Anap-W434F, superposition of the TMR and Anap fluorescence time courses revealed a delayed onset for Anap (Fig. 5G). This was best observed in R387Anap-W434F channels where it amounted to 1.40 ± 0.38 ms at 0 mV (Fig. 5B). The delay in K390Anap-W434F channels was slightly shorter with 0.93 ± 0.37 ms at 0 mV (Fig. 5C), which was likely due to the overall faster kinetics of gating and ionic currents in K390Anap-W434F. The delay was not visible in A391Anap-W434F due to decreased signal-to-noise ratio as a result of limited expression. The delay of L382Anap-W434F was within the error margin.

To ensure that the delay was not related to instantaneous C-type inactivation in the W434F mutant, we analyzed the TMR and Anap signals in the conducting L382Anap, R397Anap, and K390Anap mutants (Fig. 5D–G). Although due to smaller expression level, the signal to noise ratio was less favorable, the signals displayed the same—even slightly longer—delay for R397Anap and K390Anap, whereas the delay was absent in L382Anap.

The presence of a delayed onset in the Anap fluorescence of the C-terminal linker mutants means that at least one transition needs to occur before linker rearrangement also in the W434F background. Therefore, even though the S4–S5 linker is free to transition into its final position upon voltage sensor activation, it is not an independent movement like the voltage sensors.

The C-Terminal S4–S5 Linker Follows both Early and Late Transitions. Except for L382Anap-W434F, the QVs (Fig. 3B–E) were best fit to a two-transition Boltzmann relation, reflecting the two major charge systems Q1 and Q2 (30). There were subtle differences

![Fig. 4. Kinetic analysis of fluorescence and gating current time course. (A–D) Time constants obtained from single or double exponential fits of TMR fluorescence (green), Anap fluorescence (red), and gating currents (black) for each of the S4–S5 linker mutants during a test pulse. (E) Overlapping TMR (green), Anap (red), and charge displacement (black) for an oocyte expressing L382Anap-W434F. Arrow highlights kinetic correlation between the fast component of Anap fluorescence and charge displacement. (F) Structural view from the Kv1.2-2.1 crystal structure (3) showing the four S4–S5 linker positions in red and the distance from L382 to V234 in green in S1.](image)

![Fig. 5. Comparison of onset of TMR and Anap fluorescence signal. (A–F) Overlap of TMR (green) and Anap (red) fluorescence upon depolarizations from −90 mV. Arrows in A and D highlight the absence of a delayed onset, whereas arrows in B, C, E, and F highlight the presence of a delayed onset for Anap compared with TMR. In the boxes a vertical zoom of the curves is shown to better visualize the delay. The depolarizations were chosen with respect to the respective V_{1/2} values. In the zoomed display, the data were filtered by moving average to remove white noise. (G) Voltage dependence of the delay for all three mutants. Solid symbols indicate -W434F mutants; open symbols indicate conducting. The fit relates to the W434F data, which were obtained with better signal-to-noise ratio.](image)
among the FVs of TMR and Anap and the GV. In fact, by comparing the midpoint values of Q1, Q2, and the FVs of the three C-terminal S4–S5 linker mutants, it is tempting to conclude that Anap follows Q2, whereas TMR is closer to Q1 (Table 2). However, we would have to separate both charge movements to determine whether these subtle differences are significant. To this end, we inserted the F290A mutation into the R387Anap and K390Anap background.

F290 is situated in the S2 helix where it stabilizes the voltage sensor in the activated state, presumably by controlling the transfer of the fourth gating charge (31, 32). When mutated from phenylalanine to alanine, the early gating transitions remain nearly unaffected, whereas the final transitions shift to higher potentials. In agreement with WT-F290A channels (32), the GV curve was shifted to higher potentials when F290A was inserted into R387Anap and K390Anap (open black circles, Fig. 6 C and D) with GV midpoint values of 134.8 and 95.5 mV, respectively (Table 3).

In the W434F background, wild type (F290A-W434F) and both mutants, R387Anap-F290A-W434F and K390Anap-F290A-W434F, exhibited a biphasic GV in which ~20% of the charge activated at higher potentials (Fig. 6 A and B) (31). The second charge movement developed in the voltage range of channel opening with Q2 (Table 3). The Anap-FV also split into two components (Fig. 6 C and D). The first component (F1) remained shifted by 10–12 mV to potentials more depolarized than Q1, whereas the second component (F2) developed in the voltage range of Q2. The slight shift between Q2 and F2 is caused by the shift of the off-gating currents versus the on-gating currents (8). The Q2 had to be obtained from off-gating currents because the oocytes exhibit endogenous K+ currents at potentials more positive than +50 mV (33, 34) (black arrows, Fig. 6 A, B, and E), making it challenging to determine the displaced charge during depolarization in that voltage range. The F290A data show that the C-terminal part of the S4–S5 linker rearranges at least twice during activation. The first rearrangement occurs with a voltage dependence falling between Q1 and Q2, and the second rearrangement coincided with Q2.

**Model for the Cytosolic Gating Machinery.** In this study, the fluorescent unnatural amino acid Anap was site-specifically incorporated into various positions in the S4–S5 linker of the Shaker channel. Two-color voltage clamp fluorometry was used for detection of voltage-gated fluorescence changes, allowing the dynamical study of protein rearrangements from two regions in the same protein simultaneously. Anap was successfully inserted into both the N-terminal part (L382) and the C-terminal part (R387, K390, and A391) of the S4–S5 linker. In a previous work, we already studied the movement of the cytosolic end of the VSD (V234Anap) and pore opening (H486Anap) (17). By combining these results, we are now in a position to follow the progression of the voltage-gated rearrangements throughout the voltage-gated K+ channel Shaker: the extracellular end of the VSD (A359C-TMRM), the cytosolic end of the VSD (V234Anap), the N and C termini of the S4–S5 linker (L382Anap, R387Anap, K390Anap, and A391Anap), and the cytosolic pore gate (H486Anap; Fig. 7).

We found that the VSD move independently coinciding with their gating charge movement. The cytosolic end of VSD moves faster than the external surface, but they share their voltage dependence (17). Here we found that the N terminus of the S4–S5 linker also follows the gating charge movement (Fig. 7, transition A). The C terminus of the S4–S5 linker, in contrast, moves at potentials intermediate between gating charge movement and pore opening (Fig. 2). The difference in movement of the N and C termini indicates that due to flexibility between L382 and R387, energy is briefly stored in this region until the S4–S5 linker responds by relaxing into its final state (Fig. 7, transition B). The S4–S5 linker thus constitutes the transition between the independently acting voltage sensors and the cooperative pore opening step.

Pore opening, monitored at H486, occurred in two steps (Fig. 7, transitions B and C), one of which also precedes conduction (GV) (17) (Fig. 6F). The sequence of events would thus begin with VSD activation and S4–S5 linker bending. This is followed by “tension” or energy relief in the S4–S5 linker and pre-activation of the pore and finally pore opening and C-type inactivation (Fig. 7, transition C). Although the essential step of C-type inactivation occurs in the selectivity filter (35), it has been shown to interact with the cytosolic pore gate at the helical bundle crossing (36). We found accordingly rearrangements correlating with C-type inactivation in both the N and C termini of the S4–S5 linker (Fig. 2) and the S4 (Y234Anap) (17). We verified the rearrangements at the helical bundle crossing at H486Anap and found, indeed, a slow fluorescence change correlating with entry into C-type inactivation upon prolonged depolarization (Fig. 6C). C-type inactivation thus leads to conformational changes in the cytosolic pore gate, the entire S4–S5 linker, and the VSD.

### Table 2. \(V_{1/2}\) and \(z\) values were obtained from two- or three-state Boltzmann fits of FV and GV (W434F) curves of the indicated mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>TMR FV</th>
<th>Anap FV</th>
<th>GV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{1/2}), mV</td>
<td>(z)</td>
<td>(V_{1/2}), mV</td>
</tr>
<tr>
<td>L382Anap-W434F</td>
<td>−41.5 ± 0.9</td>
<td>1.13 ± 0.03</td>
<td>−51.0 ± 0.4</td>
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<tr>
<td>R387Anap-W434F</td>
<td>−38.5 ± 0.8</td>
<td>2.27 ± 0.10</td>
<td>−26.1 ± 0.4</td>
</tr>
<tr>
<td>K390Anap-W434F</td>
<td>−48.1 ± 0.2</td>
<td>2.58 ± 0.05</td>
<td>−40.5 ± 0.5</td>
</tr>
<tr>
<td>A391Anap-W434F</td>
<td>−42.9 ± 0.4</td>
<td>2.36 ± 0.04</td>
<td>−39.4 ± 0.2</td>
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### Table 3. \(V_{1/2}\) and \(z\) values obtained from single or three-state Boltzmann fits of FV, GV, and GV curves of F290A mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Anap FV</th>
<th>GV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A_1/A_2)</td>
<td>(V_{1/2}) ((\tau)), mV</td>
</tr>
<tr>
<td>F290A-R387Anap-W434F</td>
<td>0.4</td>
<td>−24.3 ± 5.1</td>
</tr>
<tr>
<td>F290A-K390Anap-W434F</td>
<td>0.7</td>
<td>−49.5 ± 0.7</td>
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</table>
Most significantly, the behavior of the S4–S5 linker is altered in the presence of accelerated C-type inactivation (W434F) (29). Here we found that the shift in the voltage dependencies between voltage sensor movement and S4–S5 linker rearrangements disappears in W434F (Fig. 3). However, the S4–S5 linker rearrangements are temporally delayed versus the voltage sensor movement, indicating that they do occur subsequently. In contrast, the movement of the cytosolic pore gate (H486) is not affected by W434F and still shows two transitions that occur at potentials more depolarized than gating charge movement (17). These results suggest that the S4–S5 linker, although hindered to enter its final state in the closed WT channel, is no longer hindered to directly enter its final conformation in the presence of accelerated C-type inactivation. Because pore gate opening is not altered in W434F, it suggests that it is C-type inactivation, which evens the path to the final state.

In the proposed model in Fig. 7, the voltage sensor and S4–S5 linker would transition directly into their relaxed state upon opening and inactivation (Fig. 7, Right, transition B), whereas we find the same two transitions at the C-terminal S6 (Fig. 7, transitions C and D). Although we detected the movements of the S6 in the presence of W434F, we cannot measure the conducting state of the pore and thus cannot be certain whether the two transitions are identical to the conducting mutant.

**Discussion**

Our findings provide us with a detailed picture about the rearrangements following membrane depolarization in voltage-gated K^+^ channels including the voltage sensing domain, S4–S5 linker, and cytosolic pore gate. Temporally correlating the signals with electrical signals from gating charge movement and ion conduction allowed us to establish a sequence and kinetics of the gating mechanism at the cytosolic side of voltage-gated K^+^ channels.

The mechanism underlying the fluorescence changes is quenching of the fluorophore most likely by the surrounding protein. As such, we probe movement of the labeled site relative to the quenching group. We therefore have to take more than one position into account when assigning movements to the fluorescence changes. Due to the α-helical structure of the S4–S5 linker, for instance, the amino acid residues of K390 and A391 point into different directions but still display very similar fluorescence signals, indicating that the changes are caused by movement of the S4–S5 linker rather than its environment.

It was remarkable that the C-terminal S4–S5 linker and the cytosolic pore gate react differently to the presence of W434F. Although the S4–S5 linker is free to move with the voltage sensor in W434F, the cytosolic pore gate still opens at more depolarized potentials. Our data suggest that despite uncoupling of S4–S5 linker movement from S6 movement in the presence of W434F, the channel still opens normally. This seems to contradict our current understanding of electromechanical coupling, according to which the annealing of the S4–S5 linker and the complementary C-terminal S6 leads to coinciding movements (6, 8, 9–12). A mechanism similar to what we see here, where uncoupling of the S4–S5 linker from S6 is linked to pore inactivation, has been suggested for closed state inactivation in Kv4 channels (37), and it will thus be interesting to investigate S4–S5 linker movement in Kv4.

An important feature in the C-terminal S4–S5 linker rearrangements was that we still observed a slow conformational change even in the presence of W434F. This component was sensitive to 4-AP but occurred at potentials more depolarized than pore opening. It might be an indication that the S4–S5 linker, despite not entering a “strained” state in presence of W434F, only enters its final state after pore opening.

Considering the conservation of the electromechanical coupling for many voltage-gated ion channels as well as the occurrence of C-type inactivation for many voltage-gated potassium channels, we expect the mechanism described here to be conserved throughout for those voltage-gated ion channels that contain a sizeable S4–S5 linker. In this context, also the role of auxiliary subunits of Kv channels will be of interest. The cytosolic β subunits bind to the lower T1 domain (2) and could only indirectly influence the

![Image](image_url)
S4–S5 linker position. In contrast, KChip in Kv4 channels bind laterally to the T1 domain (38) and could therefore influence directly the S4–S5 linker region. How far cytosolic auxiliary subunits might influence the mechanisms described here will have to be confirmed in further studies.

Recent structures of the EAG and HerG channels (39, 40) revealed that these channels have very short S4–S5 linkers. Consequently, their voltage sensing domain directly neighbors the S5–S6 pore domain of the same subunit, as opposed to the neighboring one as observed in Shaker-like Kv channels (“do-

main swapping”). In the Shaker-like Kv channels, the linker anneals to the C-terminal S6 but also forms a cuff around the pore and “presses” it inward (20). This is not possible in the HerG and EAG channels, and it will be interesting to study in how far our findings here are valid in those channels. It had been suggested that in these channels, the direct interface between voltage sensor and S5–S6 plays a more important role (40–42).

Because both EAG and HerG channels display a depolarizing shift between the voltage dependencies of charge movement and pore opening, just as Shaker-like Kv channels (43–45), the shorter S4–S5 linker does not seem to influence the independent movement of the first voltage sensors. However, although HERG shows very rapid C-type inactivation, EAG channels do not inactivate. One could thus speculate that the longer linker in Shaker-like Kv channels is required for the delay between activation and C-type inactivation. This would suggest that these channels rather follow the transitions of the W434F mutant (Fig. 7, Right). Only a detailed study will be able to establish this.

Methods

Xenopus Oocyte Injection. All mutations were introduced into the Droso-

sophila Shaker H4 gene in the pBSTA vector with a deletion of amino acids 6–46 to remove N-type inactivation (19). For Anap incorporation, 9.2 nL of 0.1 ng/nL cdNA encoding the tRNA/AnapRS pair (pAnap) (17, 22) was injected into the nucleus of Xenopus laevis oocytes 6–24 h before coinjection of 23 nL 1 mM Anap (custom synthesis TCRS-170; Abzena TCRS) and 35 ng in vitro transcribed RNA. All steps during and after Anap injection were performed under red light to avoid bleaching. Oocytes were then incubated for 1–3 d at 18°C in Barth’s solution supplemented with 5% horse serum.

Electrophysiology. Voltage clamp was performed with a CA-1B amplifier (Dagan). Currents were recorded in the cut-open oocyte voltage-clamp configuration as described (8) and analyzed by using GPatch (Department of Anesthesiology, University of California, Los Angeles). Linear capacitance currents were subtracted online using the P11 protocol (46) or offline using a negative pulse from −100 to −120 mV (F290A mutants). For ionic recordings the external solution contained, in mM, 5 KOH, 110 NMDG, 10 Hepes, and 2 Ca(OH)₂, and the internal solution contained, in mM, 115 KOH, 10 Hepes, and 2 EDTA. For gating current recordings, KOH was replaced by NMDG. For blocking experiments, 5 mM NMDG or KOH was replaced by 5 mM 4-

aminopyridine (4-AP), and the command potential was held at 0 mV for 5 s before recordings. All solutions were adjusted to pH 7.1 with methanesulfonic acid (Mes). Conductance (G) was calculated from the steady state currents (I) via G = I(V − Vrev), where Vrev is the reversal potential. Conductance–voltage relationships (GV) were fitted to a Boltzmann relation of the form G/Gmax = (1 + exp(−zF(V − V1/2,1/2))/RT)−1. The total gating charge (Q) was calculated from the gating currents by integration, and charge–voltage relationships (QV) were fitted to a three-state Boltzmann relation of the form Q/Qmax = A1(1 + f1 − g1) + A2(1 + f1 + g1)−1, with f1 = 1 + exp(zF(V − V1/2,1/2))/RT and g1 = 1 + exp(−zF(V − V1/2,1/2))/RT. Because the gating charge was normalized to its maximal value, A2 equals 1.

Voltage Clamp Fluorometry. For two-color VCF, oocytes were incubated in 5 mM TMR-maleimide in depolarizing labeling solution [in mM, 115 KOH, 10 Hepes, and 2 Ca(OH)₂, pH 7.1, with Mes] for 20 min at room temperature, before recordings. The oocytes were washed three times in labeling solution to remove excess dye. Fluorescence intensities were recorded with a photo-

todiode detection system (Photomax 200; Dagan) using ex-545/25, dc-570, em-605/70 (TMR) or ex-377/50, dc-409, and em-470/40 (Anap) filter sets. Fluorescence–voltage (FV) relationships were fitted to a single or three-state Boltzmann relation of the form ΔF/ΔFmax = (1 + exp(−zF(V − V1/2,1/2))/RT)−1 and ΔF/ΔFmax = A1(1 + f1 − g1) + A2(1 + f1 + g1)−1, respectively, with f1 = 1 + exp(−zF(V − V1/2,1/2))/RT and g1 = 1 + exp(−zF(V − V1/2,1/2))/RT. Because the gating charge was normalized to its maximal value, A2 equals 1.


