Fine-tuning of Voltage Sensitivity of the K$_{\text{v}}$1.2 Potassium Channel by Interhelix Loop Dynamics*

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Background: Potassium channels change conformation in response to transmembrane voltage.

Results: The loop connecting the voltage sensing helix to an adjacent helix affects the voltage sensitivity.

Conclusion: Loop length and charge distribution on the interacting surfaces of the voltage sensor and pore domain are responsible for this effect.

Significance: Variation in loop length and composition is a factor in determining the voltage sensitivity of voltage-gated channels.

Many proteins function by changing conformation in response to ligand binding or changes in other factors in their environment. Any change in the sequence of a protein, for example during evolution, which alters the relative free energies of the different functional conformations changes the conditions under which the protein will function. Voltage-gated ion channels are membrane proteins that open and close an ion-selective pore in response to changes in transmembrane voltage. The charged S4 transmembrane helix transduces changes in transmembrane voltage into a change in protein internal energy by interacting with the rest of the channel protein through a combination of non-covalent interactions between adjacent helices and covalent interactions along the peptide backbone. However, the structural basis for the wide variation in the V$_{50}$ value between different voltage-gated potassium channels is not well defined. To test the role of the loop linking the S3 helix and the S4 helix in voltage sensitivity, we have constructed a set of mutants of the rat K$_{\text{v}}$1.2 channel that vary solely in the length and composition of the extracellular loop that links the S3 and S4 helices. We evaluated the effect of these different loop substitutions on the voltage sensitivity of the channel and compared these experimental results with molecular dynamics simulations of the loop structures. Here, we show that this loop has a significant role in setting the precise V$_{50}$ of activation in K$_{\text{v}}$1 family channels.

Voltage-gated potassium (K$_{\text{v}}$) channels are transmembrane proteins essential for the electrical activity of excitable cells (1). K$_{\text{v}}$ proteins, similar to other voltage-gated ion channels, convert changes in membrane potential into useful work (2) opening and closing the ion permeation pathway (3). Four α subunits assemble to form a functional channel, with the pore domain (PD) regions interlocking in the center to form the ion pathway and potassium selectivity filter (4, 5). The surrounding voltage-sensing domains (VSDs) contact the PD from an adjacent subunit (6) but are otherwise largely surrounded by membrane lipids (4, 7, 8).

Each K$_{\text{v}}$ channel subunit consists of six transmembrane helices, the linker sequences that connect them, and N- and C-terminal modulatory domains. Helices S1 through S4 constitute the VSD, and helices S5, S6, and the intervening re-entrant pore loop make up the PD. The VSD response to changes in membrane potential originates from the S4 helix, which contains four to seven positive amino acids (depending on the channel type) and translocates through the changing electric field of the membrane to drive the opening and closing of the channel (9–11). Under depolarizing conditions, each VSD traverses a number of pre-opening conformations before the channel undergoes a final, cooperative step that opens the pore (12–14).

A combination of three-dimensional crystal structures (5, 8) and molecular dynamics simulations (15–19) support a “sliding helix” model of K$_{\text{v}}$ activation. The predominant feature of this model is a significant translation and rotation of the S4 helix relative to the rest of the VSD. The movement of S4 is constrained by and acts on the rest of the channel by non-covalent interactions with adjacent helices in the VSD and by covalent interactions with the extracellular loop that links the S3 and S4 helices as well as the intracellular loop that links the S4 and S5 helices. Thus, when the force on the S4 helix changes with membrane potential, the overall conformation of the channel protein changes. The sum of these energetic interactions within the protein and between the protein and the lipid bilayer is ultimately responsible for setting the V$_{50}$ of the channel, the voltage at which half of the channels are in an open state and half are in a closed state.

Although there is a growing consensus regarding the structure of K$_{\text{v}}$ channels in the open and closed states, the mechanisms by which voltage sensitivity is modulated remain unclear. Even within the same subfamily, V$_{50}$ can vary widely between K$_{\text{v}}$ channels. In the K$_{\text{v}}$1 group, for example, published V$_{50}$ val-

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3 The abbreviations used are: K$_{\text{v}}$, voltage-gated potassium; V$_{50}$, voltage at which conductance is activated to 50% of maximum; VSD, voltage-sensing domain; PD, pore domain.
ues range from −46 mV in a lobster channel (20) to +22 mV in a jellyfish channel (21). Human channels K_1.1 and K_1.2 share 80% sequence identity and yet differ in voltage sensitivity by 25 mV, with \( V_{50} \) values of −30 mV and +5 mV, respectively (22). These differences in \( V_{50} \) despite sequence conservation indicate that precise control of \( V_{50} \) ultimately resides in the more variable, and generally less studied, regions such as interhelical loops.

Besides the membrane lipid head groups (23, 24) and acidic residues in adjacent helices (25, 26), the S3/S4 linker has been implicated in the regulation of voltage sensitivity in K_1 channels. In a cnidarian K_1 channel, the role of one of the S2 acidic residues in setting the \( V_{50} \) can be modified by the constraints of a short S3/S4 loop (27). Sequential truncation of the S3/S4 loop in the Shaker channel also changes voltage sensitivity and gating kinetics (28, 29); however, this simple reduction of the wild type loop length could have several confounding effects that cannot be distinguished in a single truncation mutagenesis series.

In this study, we have varied both the length and composition of the S3/S4 loop in the K_1.2 channel to evaluate their contributions to the overall voltage sensitivity of the channel. The experimental results from the mutant channels were combined with molecular dynamics simulations of the same loops constrained by models of open and closed states of the channel. We found that both the length and the composition of the loop are responsible for an energetic constraint on the transition between the opened and closed states. We conclude that the highly variable range of lengths and compositions of S3/S4 linkers found in nature is one factor in the quantitative evolution of voltage sensitivity in the K_1 family of voltage-gated cation channels.

**EXPERIMENTAL PROCEDURES**

**Channel Construction and Expression**—Full-length mouse K_1.2 was purchased (OpenBiosystems, Huntsville, AL) and subcloned into the *Xenopus laevis* oocyte expression vector pXT7 (30); note that mouse and rat K_1.2 have identical amino acid sequences. To create loop variants, two opposing BbsI sites were introduced into the S3/S4 loop by overlapping PCR mutagenesis to create a construction vector. Annealed oligonucleotides with the correct sequence and overhangs were ligated into the BbsI-digested K_1.2 vector. Sequences of all plasmids were confirmed by Sanger sequencing. Plasmids were linearized with XbaI (Promega, Fitchburg, WI) for *in vitro* mRNA transcription with T7 RNA polymerase (Ambion, Austin, TX). Injection volumes of the *in vitro*-transcribed mRNA were optimized to produce between 4 and 18 \( \mu \)A maximal current, as currents larger than ~20 \( \mu \)A cause local distortions in membrane potential (31) and because some channels have been shown to be sensitive to high extracellular \([K^+]\) (32). Oocytes were incubated at 18 °C in modified Barth’s medium (33) prior to experiments. During experiments, oocytes were bathed in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2, 1 mM MgCl_2, 5 mM HEPES, 1 mM diisothiocyanatostilbene-2,2’-disulfonic acid to block native chloride currents (34), pH 7.4.

**Electrophysiology**—Ionic currents were recorded 24–48 h post-injection from *X. laevis* oocytes at room temperature using two-electrode voltage clamp as described previously (35). Traces were filtered at 1 kHz and sampled at 10 kHz with P/4 leak subtraction. Cells were held at a potential of −90 mV and test pulses were 50-ms in duration. Conductance was derived from tail current amplitudes fitted with double exponential decay functions. Individual G-V curves were normalized and fitted to a Boltzmann equation of the form (36).

\[
G/G_{\text{max}} = \frac{1}{1 + \exp ((V_m - V_{50})/b)^{4}}
\]

The \( V_{50} \) in this equation is of one subunit; channel \( V_{50} \) values were calculated by solving for \( V_m \) when \( G/G_{\text{max}} = 0.5 \). Data were collected and analyzed using pClamp (version 9; Molecular Devices, Sunnyvale, CA) and SigmaPlot (version 12; Systat Software, Inc., Point Richmond, CA).

**Molecular Models**—The open state models for rat K_1.2 are based on the Protein Data Bank code 3LUT refinement (37) of the crystal structure (5). The closed state models are based a structure inferred by Jensen *et al.* (16), based on the structure of a K_1.2/K_2.1 chimera; the K_2.1 segment from that chimera was replaced with the homologous K_1.2 sequence using SWISS-MODEL (38). The structures of the various sequence variants were obtained by using SWISS-MODEL for homology modeling and PyMOL (39) for simple mutagenesis; as such, these models should be considered as heuristic.

**Molecular Dynamics Simulations**—Peptide structures were constructed in Protein Data Bank format using PyMOL (39), with the N terminus capped with an acetyl group and the C terminus capped with an amine group. Protein Data Bank files were processed using the GROMACS suite of programs (40), using the AMBER99-SB force field to produce a peptide in an aqueous environment with NaCl concentration of 96 mM. An initial ensemble of conformations was generated by performing simulation under constant pressure and temperature (NPT) conditions (after appropriate pre-equilibration) for 100 ns. 10 conformations at 10-ns intervals were then selected and used as starting points for independent free energy simulations. For free energy calculations, each selected conformation was reprocessed into an aqueous environment with NaCl concentration of 96 mM, energy minimized, equilibrated under a constant volume and temperature regime followed by an NPT regime, and simulated under NPT conditions for 2 ns under an end-to-end distance constraint ranging from 0.5 to 2.5 nm in 20 equal increments, collecting data necessary for free energy calculations. The results of the simulations were processed to obtain estimates of the free energy differences between adjacent distance increments using the Bennett Acceptance Ratio (41) as implemented in the g_bar program from the GROMACS suite.

**RESULTS**

**Design and Construction of Mutant Channels**—The structure of the S3 and S4 helices in the closed (Fig. 1A) (16) and open (Fig. 1B) (37) state models show that the extracellular ends of the helices are translocated away from each other during channel closing. We wanted to determine whether the distance between the ends of the helices, and therefore the length of the linker, was the only constraint on the Gibbs free energy of activation (\( \Delta G_{\text{open}} \)) or if the loop was interacting with the rest of the channel to modulate \( V_{50} \).
Effects of Interhelical Loops on Kv1.2 Channel Activity

We prepared 39 mutants of the mouse Kv1.2 channel (accession no. NM_008417), which is identical in amino acid sequence to the rat Kv1.2 channel (hereafter we will refer to this as a rat channel for consistency with other published results using the same amino acid sequence). Fig. 1C illustrates the mutagenesis where the 13 amino acids from the middle of the 18-amino-acid-long S3/S4 loop were replaced with the following: (i) homoloops, homopolymers of glutamate (Glu), glycine (Gly), and serine (Ser) ranging in length from 2 to 10 amino acids (except for Gly, where G9 was the longest loop); (ii) heteroloops, E7, G7, and S7 loops combined with three C-terminal residues consisting of E9, G9, S9, or glutamine-glutamine-alanine (QQA, the wild type C-terminal three amino acids) in all combinations; (iii) WT/endloops - wild type Kv1.2 linker with the C-terminal QQA replaced with E9, G9, or S9; or (iv) TEV-loop, with a sequence that contained the target site for TEV protease. The mutants expressed in oocytes all mediated delayed rectifier currents, with distinct V50 values and rates of activation and deactivation.

ΔG_{open} of Loopless Channel (ΔG_{core}) Reveals S3/S4 Loop Stabilizes the Open State—The Gibbs free energy of activation (ΔG_{open}) of an intact channel can be decomposed into the free energy of the conformational change in the S3/S4 loops (ΔG_{loops}) plus the free energy of the conformational change of the rest of the channel (ΔG_{core}) plus an unknown interaction energy (ΔG_{inter}), that represents the difference in non-covalent interaction energy between the loop and the rest of the channel in the open and closed states.

$$\Delta G_{\text{open}} = \Delta G_{\text{core}} + \Delta G_{\text{loops}} + \Delta G_{\text{inter}} \quad (\text{Eq. 2})$$

To characterize the unknown interaction energy for the various mutant constructs, we first determined ΔG_{core} experimentally by cleaving the S3/S4 loops with TEV protease (i.e., by making ΔG_{loops} + ΔG_{inter} equal to zero) and determining the V50 of this modified channel (Fig. 2A).

The V50 for the TEV digested loop mutant was −7.5 ± 0.9 mV, which was significantly different from the wild type channel (−13.3 ± 1.7 mV) and from the TEV mutant channel that was not digested with the TEV enzyme (−14.7 ± 1.4 mV) (Fig. 2A).

The free energy of opening was calculated as shown in Equation 3,

$$\Delta G_{\text{open}} = 13V_{50}F \quad (\text{Eq. 3})$$

where F is Faraday’s constant and 13 represents the total gating charge that translocates during channel opening in rat Kv1.2 (42).

This calculation yielded a ΔG_{core} of −9.4 ± 1.1 kJ/mol for the TEV-digested TEVloop mutant. Subtraction of ΔG_{core} from the ΔG_{open} of the wild type channel yields a net difference on ΔG of −7.3 ± 2.5 kJ/mol (Table 1), implying that each of the four wild type loops in the Kv1.2 channel stabilizes the open state to the extent of ~1.8 ± 0.6 kJ/mol.

Length and Composition Modulate ΔG_{open} in Loop Mutants—Changing the length and character of the S3/S4 loop shifted the midpoint of steady-state activation left or right on the voltage axis but did not greatly affect the slope in the linear portion of the sigmoid curve (Fig. 2, B–F).

The effects of loop length and composition on V50 were pronounced among the homoloop mutants (Fig. 2, B–D). Loops with inserts consisting of only two residues (E6, G7, and S7) had extremely positive V50 values, showing shifts of approximately +40 to +55 mV compared with wild type. As the loop inserts were lengthened V50 values for all homoloop channels became more negative, approaching and sometimes becoming more hyperpolarized than that of the wild type channel. Glutamate loops produced the most negatively shifted V50 values; E6 through E10 had significantly more negative V50 values (−19 ± 2 mV for E6 and −28.3 ± 1.5 mV for E10) than wild type Kv1.2 (−13.3 ± 1.8 mV). Interestingly, G4 and S4 also manifested significantly hyperpolarized V50 values compared with wild type, approximately −24 and −22 mV, respectively.

Because current models of the closing process involve the S4 helix moving a substantial distance in the intracellular direction relative to the rest of the VSD, thus pulling the C terminus of the S3/S4 loop into the extracellular vestibule of the VSD, we also constructed loop mutants in which the last three residues of the loops were swapped between all of E10, G10, S10, and wild type, to test whether changing the C-terminal end of the loop would alter ΔG_{open} by virtue of being pulled into a position in which it would directly interact with other VSD residues. The relative effects of varying just the C-terminal three amino acids in the
loop insert on the conductance-voltage curve are shown in Fig. 2, E and F. Clearly, the composition of the C-terminal segment of the loop sequence has a significant effect on the channel $V_{50}$ and thus the energy difference between open and closed states.

Overall, the loop variants produced $\Delta G_{\text{open}}$ values ranging from $-35.7$ kJ/mol in $E_8$ channels to $+53.8$ kJ/mol in $S_2$ channels (Table 1). Subtracting $\Delta G_{\text{core}}$ from $\Delta G_{\text{open}}$ produced values ranging from $-26.3$ to $+63.2$ kJ/mol per channel. Thus, replacing each wild type S3/S4 linker with eight glutamate residues stabilized the open state by $\sim 6.5$ kJ/mol/loop, while replacing it with two serine residues destabilized the open state by $\sim 15.8$ kJ/mol/loop. Recalling Equation 2, these energies represent the sum of $\Delta G_{\text{loop}}$ and $\Delta G_{\text{inter}}$. To find $\Delta G_{\text{inter}}$, we estimated $\Delta G_{\text{loop}}$ through molecular dynamics simulations.

**Molecular Dynamics Simulations of Loops**—The first step in estimating $\Delta G_{\text{loop}}$ was to simulate untethered individual loop peptides in water and determine the Gibbs free energy as a function of loop end-to-end distance (Fig. 3). We then assumed the closed (2.02 nm) and open (1.37 nm) state distances predicted by recent models (Fig. 1, A and B) and found the free energy difference between those points on each curve. This difference represented $\Delta G_{\text{loop}}$ in all subsequent energetic calculations.

In addition, each curve had a single minimum corresponding to the most energetically favored end-to-end distance for that loop. We compared these favored end-to-end distances to the $V_{50}$ values for each loop type to see whether these minima correlated with voltage sensitivity. Surprisingly, the loops with energy minima at end-to-end distances close to that observed in the three-dimensional crystal structure, which is considered typical of the open state ensemble of conformations, did not correspond to the most negative $V_{50}$ value. In fact, these loops yielded channels with some of the most positive $V_{50}$ values. Among all loop mutants, there was no significant correlation of $V_{50}$ with the end-to-end distance of minimum energy ($R^2 = 0.165$ on linear regression of the data in Table 1).

$\Delta G_{\text{inter}}$ of Loop Mutants Demonstrates Collateral Loop Interactions—The difference between $\Delta G_{\text{open}}$ and the sum of $\Delta G_{\text{core}}$ and $\Delta G_{\text{loop}}$ is attributable to collateral interactions between the loop and the rest of the channel (\(\Delta G_{\text{inter}}\)). Thus, a non-zero $\Delta G_{\text{inter}}$ value indicates that the energy difference between open and closed states is not simply the sum of the internal energy of the channel core and the end-to-end energy difference of the loops.

Fig. 4A shows the $\Delta G_{\text{inter}}$ values as a function of loop length for the homoloops series. Loop insertions of two and three have large positive values of $\Delta G_{\text{inter}}$ for all three amino acids tested, which is a reflection of the extremely positive $V_{50}$ values (and thus $\Delta G_{\text{open}}$) in these channels. Glycine loop inserts of lengths 5 through 9 have $\Delta G_{\text{inter}}$ values essentially equal to zero, whereas glutamate loop inserts of lengths 5 through 10 have monotonically increasingly negative $\Delta G_{\text{inter}}$ values that plateau at approximately $-47$ kJ/mol. This implies a strong interaction between glutamate loops and the rest of the channel. Serine inserts, on the other hand, tend to have small $\Delta G_{\text{inter}}$ values.
Effects of Interhelical Loops on $K_{v1.2}$ Channel Activity

**Table 1**

| Loop | $\Delta G_{\text{inter}}$ | $\Delta G_{\text{loops}}$ | $\Delta G_{\text{loops}} + \Delta G_{\text{inter}}$ | $\Delta G_{\text{loops}}$ | $\Delta G_{\text{loops}}$ | $\Delta G_{\text{inter}}$ |
|------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| TEV uncut | 9 | −14.7 | 1.4 | −9.0 | 2.1 | −9.1 | 2.1 | 0.1 | 3.0 |
| WT | 8 | −13.3 | 1.8 | −7.3 | 2.5 | 3.9 | 1.1 | 11.2 | 2.8 |
| G$_3$ | 11 | 42 | 2.7 | 62.1 | 3.6 | 19.7 | 1.3 | 81.7 | 3.8 |
| G$_7$ | 7 | −15.2 | 1.5 | −9.7 | 2.2 | −12.1 | 0.9 | 2.5 | 2.4 |
| G$_{6}$ | 11 | −23.8 | 1.2 | −20.4 | 2.0 | 19.7 | 1.3 | 19.5 | 2.0 |
| G$_7$ | 10 | −13.9 | 1.5 | −8.0 | 2.2 | −4.2 | 0.9 | −3.9 | 2.4 |
| G$_{6}$ | 9 | −10.7 | 1.3 | −4.0 | 2.0 | −2.8 | 0.4 | −1.2 | 2.0 |
| G$_7$ | 9 | −10.7 | 1.2 | −4.0 | 1.9 | −0.7 | 0.6 | −3.3 | 2.0 |
| G$_{6}$ | 9 | 48.6 | 1.2 | 12.6 | 1.9 | 13.3 | 0.3 | 1.3 | 1.9 |
| G$_{6}$ | 9 | 9.2 | 0.8 | 2.1 | 1.5 | 1.2 | 0.6 | 2.1 | 1.6 |
| S$_2$ | 9 | 42.9 | 7.4 | 63.2 | 9.4 | 27.4 | 1.9 | 90.6 | 9.5 |
| S$_5$ | 8 | −3.3 | 1.5 | 5.3 | 2.2 | −35.6 | 1.5 | 40.9 | 2.7 |
| S$_4$ | 11 | −22.5 | 1.1 | −18.8 | 1.8 | −11.7 | 1.9 | −7.1 | 2.6 |
| S$_3$ | 11 | −16.1 | 1.7 | −10.8 | 2.0 | −14.0 | 1.2 | 3.6 | 2.7 |
| S$_6$ | 9 | −7.7 | 1.4 | −0.3 | 2.1 | −9.3 | 1.2 | 9.1 | 2.4 |
| S$_7$ | 9 | −14.3 | 2.7 | −8.5 | 3.6 | 1.9 | 0.8 | −10.4 | 3.7 |
| S$_8$ | 9 | −14.6 | 2.7 | −8.9 | 3.6 | 8.8 | 0.5 | −17.7 | 3.6 |
| S$_9$ | 9 | −14.2 | 1.9 | −8.4 | 2.6 | −8.2 | 1.0 | −10.2 | 2.8 |
| S$_{10}$ | 10 | −13.7 | 2.1 | −7.8 | 2.9 | 1.9 | 0.8 | −9.7 | 3.0 |
| E$_2$ | 12 | 29.1 | 1.5 | 45.9 | 2.2 | −25.2 | 2.6 | 71.1 | 3.4 |
| E$_3$ | 9 | −1.6 | 1.4 | 7.4 | 2.1 | −24.9 | 2.1 | 32.3 | 2.9 |
| E$_4$ | 8 | −9.2 | 1.5 | −5.1 | 2.2 | 8.8 | 1.7 | −10.9 | 2.8 |
| E$_5$ | 7 | −13.9 | 1.5 | −8.0 | 2.2 | 9.2 | 1.5 | −17.2 | 2.7 |
| E$_6$ | 9 | −19.8 | 2.1 | −15.4 | 2.8 | 13.8 | 0.7 | −29.2 | 2.8 |
| E$_7$ | 9 | −22.1 | 2.1 | −18.3 | 1.9 | 19.2 | 1.1 | −37.5 | 2.2 |
| E$_{10}$ | 9 | −28.5 | 2.1 | −26.3 | 2.8 | 20.9 | 0.8 | −47.3 | 3.0 |
| E$_{10}$ | 9 | −28.3 | 1.8 | −26.1 | 2.5 | 20.6 | 0.9 | −46.6 | 2.7 |
| WT-G$_3$ | 9 | 48.6 | 1.2 | 11.1 | 2.3 | 5.7 | 0.6 | −16.8 | 2.4 |
| WT-G$_3$ | 11 | −21.6 | 1.1 | −17.7 | 1.8 | 12.5 | 0.5 | −39.9 | 1.9 |
| WT-E$_2$ | 9 | −22.9 | 0.6 | −19.3 | 1.4 | −2.8 | 1.6 | −16.6 | 2.1 |
| G$_{3}$-QQA | 10 | 1.8 | 1.2 | 11.7 | 1.9 | −8.0 | 1.0 | 19.7 | 2.1 |
| G$_{5}$-QQA | 9 | −13.6 | 1.6 | −7.7 | 2.3 | −6.2 | 0.8 | −1.5 | 2.4 |
| G$_{5}$-QQA | 10 | −19.9 | 0.9 | −15.6 | 1.6 | 19.7 | 0.7 | −17.5 | 1.7 |
| E$_{3}$-QQA | 10 | −23.3 | 1.4 | −19.8 | 2.1 | 20.9 | 0.5 | −40.7 | 2.2 |
| E$_{3}$-QQA | 8 | −24.5 | 1.7 | −21.3 | 2.4 | 16.7 | 0.5 | −38.0 | 2.5 |
| G$_{5}$-QQA | 8 | ND | ND | ND | ND | ND | ND | ND | ND |
| S$_{7}$-QQA | 10 | 0.1 | 1.2 | 9.5 | 1.9 | 1.7 | 1.0 | 7.4 | 2.1 |
| S$_{7}$-QQA | 12 | −12.9 | 0.9 | −6.8 | 1.6 | −2.3 | 0.8 | −4.4 | 1.8 |
| S$_{7}$-E$_3$ | 9 | −17.3 | 1.1 | −12.3 | 1.7 | −2.0 | 1.2 | −10.3 | 2.1 |

with a periodic variation in effect that decreases in amplitude as the length of the insert increases.

To determine the contributions of two parts of the S3/S4 loop, that which is pulled into the VSD vestibule upon channel closing and that which remains in a relatively unordered conformation outside the core of the VSD in the closed state, to the energetics of channel opening and closing, we evaluated the heteroloops and WT/endo loops series of mutants (Fig. 4B). The range in $\Delta G_{\text{inter}}$ values among the mutants indicates that both the C-terminal end of the loop, which translocates into the VSD (Fig. 1A), and the N-terminal end interact with the channel to modulate voltage sensitivity.

The negative value of $\Delta G_{\text{inter}}$ for wild type (Fig. 4B, WT, blue bar) indicates that the native loops interact with the rest of the channel to destabilize the closed state (or stabilize the open state, but for simplicity from this point we will refer only to the effects on the closed state). Altering the three C-terminal residues to glycine, serine, or glutamate increases the interaction energy and further stabilizes the closed state, suggesting that the wild type C-terminal sequence is stabilizing the closed state relative to the other C-terminal tripeptide sequences.

The importance of the three C-terminal residues is also apparent in the glycine series of heteroloops. The $G_3$ loop ($G_6$ loop plus $G_3$ C terminus) has essentially no energetic interactions outside of the constraints imposed by the change in end-to-end distance between the open and closed state (Fig. 4A). However, when the C-terminal three glycine residues are replaced by the corresponding residues from the wild type loop sequence (QQA), there is an approximately +20 kJ/mol positive interaction energy, stabilizing the closed state (Fig. 4B). Substituting three glutamate residues in the same location yields an approximately −18 kJ/mol $\Delta G_{\text{inter}}$ destabilizing the closed state. A similar trend can be observed in the serine series of heteroloops. Thus, the interaction of the portion of the S3/S4 loop that translocates into the VSD on channel closing can stabilize or destabilize the closed state depending on the identity of the side chains.

Analysis of $\Delta G_{\text{inter}}$ also shows that the N-terminal end of the loop has a significant energetic interaction with the rest of the channel (Fig. 4B). For example, when the three C-terminal residues are the wild type QQA (Fig. 4B, blue bars), changing the N-terminal seven residues has a large effect on $\Delta G_{\text{inter}}$. Replacing the N-terminal residues with glycine or serine strongly stabilizes the closed state, whereas replacing them with glutamate strongly destabilizes the closed state. In fact, all loops with seven glutamate residues at the N terminus have highly negative $\Delta G_{\text{inter}}$ values (ranging from −38 to −47 kJ/mol), virtually independent of the C-terminal three residues, further supporting an interaction of the amino-terminal of the S3/S4 linker with the rest of the channel.
Moreover, the value of $\Delta G_{\text{inter}}$ is not simply a sum of effects of the two parts. For example, the C-terminal S3 (Fig. 4B, green bars) has little or no effect when compared with G3 (Fig. 4B, red bars) when placed with the G7 N-terminal sequence, whereas in the context of the wild type and S7 N-terminal sequences, C-terminal S3 causes a substantially more negative $\Delta G_{\text{inter}}$ (i.e. destabilizes the closed state) than the G3 sequence. This further supports the idea that the S3/S4 loop interacts with the rest of the channel via pathways other than the peptide bonds connecting the loop to the S3 and S4 helices, and these interactions and their impact on $V_{50}$ are a function of the channel-specific loop residue side chains.

**Electrostatic Properties near the S3/S4 Loop Influence Voltage Sensitivity**—As the glutamate loops series manifested by far the largest negative $\Delta G_{\text{inter}}$ we sought to understand the variation in $\Delta G_{\text{inter}}$ by examining the electrostatic properties near the loop in the open and closed state models. Fig. 5 illustrates the relative positions of the loop residues in the open and closed states to the nearest (within 1 nm) negatively charged residues in the rest of the channel. There are five acidic residues that are within 1 nm of at least one of the fourth, fifth, and eighth residues in the E10 loop in the closed state (Fig. 5, A and C) but are much farther from these residues in the open state (Fig. 5, B and D). This suggests that residues in the S3/S4 loop that are not interacting with the core of the VSD may be contributing to the overall energetics of activation by interacting with residues in the adjacent PD vestibule.

Given these results, the $\Delta G_{\text{inter}}$ values presented in Fig. 4 are consistent with the most recent closed state structural model of Kv1.2 (18). Glycine has no side chains and polyglycine has a low tendency to form stable secondary structures, so the interactions of the glycine series loop inserts would be the least likely to mediate non-backbone interactions with the rest of the channel. Once the polyglycine loop reaches a length of five or more and is capable of stretching across the S3/S4 gap in both open and closed conformations, the gating energy can be accounted for as the simple sum of the energy change in four loops ($\Delta G_{\text{loop}}$) plus the energy change in the loopless channel ($\Delta G_{\text{core}}$), i.e. $\Delta G_{\text{inter}} = 0$ (Fig. 4A).

The polyglutamate loops show a substantial non-backbone interaction effect on gating energy. Polyglutamate inserts of five and longer have a strong bias toward the open state reflected in their hyperpolarized $V_{50}$ values. This was unexpected for two reasons, first because the polyglutamate loops monotonically favor longer end-to-end distances over the lengths range tested (Fig. 3), and yet the most current structural models predict that the open state has a shorter end-to-end distance than the closed state. Second, if the C-terminal end of the polyglutamate chain translocates into the membrane voltage field in the closed state, depolarization would be expected to stabilize the closed state because the force of the membrane potential acting on the neg-
atively charged glutamate side chains would be opposing the force exerted by the potential on the positively charged residues in S4. However, if the transmembrane electric field is focused below this vestibule, the glutamate homopolymer would be unaffected by that potential field.

Both of the effects discussed above appear to be overwhelmed by other interactions of the polyglutamate loop with the channel. Although repulsion of glutamate side chains by residues within the VSD in the closed state appears to be part of the effect, the fact that E7G3 and E7QQA inserts are as negatively shifted as the E10 insert indicates that a major part of the effect is due to the negative charge in the first seven amino acids of the loop insert. A plausible mechanism is illustrated in Fig. 5.

**DISCUSSION**

Although loops connecting core secondary structural elements of folded protein domains, as opposed to those connecting discrete domains, are often thought of as disordered and under relatively low selective pressure, they can play a significant role in the folding and functioning of proteins (43, 44). The length of the loop or more precisely, the relative differences in free energy between ensembles of different end-to-end distances, constrain the relative motion of structural elements within domains.

The lengths of loops connecting S3 and S4 in a collection of 239 members of the K1 family of voltage-gated ion channels vary in length from six amino acids up to 34 amino acids in

![Figure 4](image1.png)

**FIGURE 4. Comparison of the ΔG_intert values (the effect of the loop that is not due to simple end-to-end distance constraints) for the different loop mutants. A, ΔG_intert as a function of loop length for the three homoloop series of insets. Error bars represent S.E. n values range from 5–12 (see Table 1). B, comparison of the effects of changing the last three residues of the loop sequence in the heteroloop and WT/endloop mutants on ΔG_intert. The identity of the N-terminal part of the loop is indicated along the horizontal axis; the identities of the C-terminal three amino acid residues in the loop are indicated by the color coding of the bars, as per the key panel in the upper right corner of the panel. Error bars represent S.E. (n = 10). All energies are expressed as kJ/mol.**

![Figure 5](image2.png)

**FIGURE 5. Illustration of proximities of S3/S4 loop residues and acidic, negatively charged residues in the rest of the channel in open and closed state models.** For clarity, only the VSD from one subunit and the PD from the adjacent subunit are shown. A and C are open state conformations of the wild type channel, B and D are closed state conformations of the E10 mutant channel. A and B are side views from within the plane of the membrane, and C and D are views from the extracellular aspect. Atoms in residues in the S3/S4 loop are rendered as green spheres, with the three C-terminal residues rendered as a lighter green than the rest of the loop. Atoms in acidic residues in the rest of the channel are rendered as red spheres. The remainder of the channel protein is rendered in white, ribbon format. The two acidic residues in the VSD that are within 1 nm of the loop in the closed state (Glu-226 and Asp-259) are labeled, except in D, where Asp-259 is hidden by the rendering of the extracellular loop. The other three acidic residues (Glu-350, Asp-352, and Glu-353, unlabelled) are in the vestibule loop of the immediately adjacent subunit.
length, with the average length being 17. In the rat Kv1.2 channel, the length is 18. Thus, the range of lengths that we have tested in these experiments, seven to 18 amino acids in length, falls within the length range that has arisen naturally during evolution.

We have used the fact that varying the loop sequence causes variation in the relative energy of the loop in the open and closed channel conformations to interrogate the energetics of the VSD of the Kv1.2 channel and explore how these loops contribute to the overall voltage sensitivity of these channels. We have combined electrophysiological data with free energies calculated from molecular dynamics simulations in a novel way that avoids some limitations inherent to protein crystallography of dynamic transmembrane proteins and those functional experiments that introduce bulky probes or other non-native, tethered molecules.

The difference between the energetics of the negatively charged loops (based on polyglutamate and wild type amino acid sequence) and the neutral loops (based on polyglycine and polyserine homopolymers) suggests an electrostatic interaction between the S3/S4 loop and other parts of the channel. The closed state model derived from unrestrained molecular dynamics simulation (16) involves two major conformational changes that affect interactions with the S3/S4 loop. 1) The S4 helix translocates ~1.5 nm in the inward direction, drawing the C-terminal end of the loop into the interior of the VSD, and 2) the VSD rotates toward the PD, such that the S4 helix is pushed into close proximity with the negative field produced by the turret of the PD.

The translocation of the C-terminal end of the S3/S4 loop into a vestibule that stabilizes the positively charged residues of S4 in the open state can explain a significant part of the deviation of the experimental channel energetics from those predicted based solely on end-to-end distance constraints. In the case of the E3 C-terminal segment electrostatic repulsion between this segment and acidic residues within the VSD is acting to destabilize the closed state, leading to a shift to more negative $V_{50}$ values. On the other hand, the QQA sequence at the C-terminal end of the wild type loop appears to stabilize the closed state, contributing to a more positive $V_{50}$ value.

Electrostatic interaction between the S3/S4 loop and the PD in the closed state but not the open state also has a role in modulation of channel activity. The wild type loops have a $\Delta G_{\text{inter}}$ value of $-11.2 \pm 2.8$ kJ/mol, but the C-terminal QQA sequence of the wild type loop tends to stabilize the closed state, i.e. contributes a positive term to $G_{\text{inter}}$ (see above). Because the remainder of the wild type loop contains three acidic residues and one basic residue, it appears that repulsion between the loop and the turret of the PD is acting to destabilize the closed state, leading to the observed value for $\Delta G_{\text{inter}}$.

Interestingly, steady-state activation of channels with loop inserts of only two or three residues is extremely right-shifted (Fig. 2) and manifests the largest positive values of $\Delta G_{\text{inter}}$ (Table 1). Yet, the most energetically stable end-to-end distance of these loop peptides in MD simulations was similar to that of the open state in the Kv1.2 crystal structure. How can these results be reconciled? Previous mutations made on the jellyfish channel jShak1, which has an extremely short S3/S4 loop, suggested that reducing the freedom of motion between S3 and S4 by lengthening the S4 helix caused steric clashing between helices in the VSD (27); this may be what is occurring in the shortest loops tested here. Because these loops are expected to exert significant force on the ends of S3 and S4 at the distances characteristic of the native closed state (2.02 nm), it appears that the shortest loop mutants in this study are forcing the channels into a different functional conformational ensemble, one that is dissimilar to the conformations identified in the experimentally determined structural models of the wild type Kv1.2 channel. These short loop constructs are similar to some of the deletion mutants constructed in the Drosophila melanogaster Shaker channel by Xu et al. (45) and by Gonzalez et al. (28), which also caused large rightward shifts in the G-V curves. In the case of these extremely short loops, however, Gonzalez et al. showed that the gating charge was reduced from 12 to ~5. It is possible that the effect of the short loops on $V_{50}$ is due to restriction of S4 motion during opening and that the $\Delta G_{\text{open}}$ values that we have calculated for channels with extremely short loops could be significantly overestimated.

The opening and closing of voltage-gated ion channels is an allosteric process (46, 47). A change in the environmental conditions of the protein (i.e. membrane potential) leads to concerted molecular rearrangements of the protein between an inactive, ion-impermeant conformational ensemble and an active, ion permeant conformational ensemble. Opening of the channel is caused by a change in outward force on the S4 voltage-sensing helix due to depolarization of the membrane potential. This initial force is transduced into internal forces that rearrange the VSD. The rearranged VSD in turn causes channel opening by pulling on the S4/S5 intracellular loop, which in turn pulls the cytoplasmic ends of the S5 and S6 helices away from the central axis of the PD, opening a path into the cytoplasm for ions that have passed through the ion selective pore. We have delineated two mechanistic elements that affect the allosteric response within the VSD (loop length and interactions of the C terminus of the S3/S4 with charged residues within the VSD) and one mechanistic element that affects the allosteric interaction between the VSD and the PD (interaction of S3/S4 loop charged residues with charged residues in the turret of the PD).

In summary, a number of factors are responsible for the effect of varying the S3/S4 loop on the voltage sensitivity of the Kv1.2 channel. The length of the loop sequence is important, with very short loops (an insert of two amino acids with five additional residues flanking the insertion site, for a total length of seven residues) of any type causing an extreme positive shift in voltage sensitivity. Longer loops (of the order of five or more residues inserted with five additional residues flanking for a total of 10 or more residues) can impose subtle energetic constraints by virtue of the difference in free energy between the closed and open state conformational ensembles. However, with the exception of glycine homopolymer inserts, the loops do not solely act in this simple manner because the nature of their side chains affects the interaction energy of the loop with the rest of the channel, and this energy varies significantly between the open and closed states. A major factor in the interaction energy appears to be interactions between the S3/S4 loop...
Effects of Interhelical Loops on Kv1.2 Channel Activity

and the negatively charged turret of the pore domain, which intensifies on channel deactivation when the movement of the majority of the VSD away from the PD rotates the S3/S4 loop into closer proximity with the PD turret. In addition, residues near the C terminus of the loop, which are part of the unordered loop in the open state, are translocated into the core VSD structure in the closed state, bringing them into proximity with acidic residues within the VSD core. The combination of these two kinds of interaction is ultimately responsible for shifting the voltage sensitivity of the channel.

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