Gating of Shaker-type Channels Requires the Flexibility of S6 Caused by Prolines*§

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The recent crystallization of a voltage-gated K⁺ channel has given insight into the structure of these channels but has not resolved the issues of the location and the operation of the gate. The conserved PXP motif in the S6 segment of Shaker channels has been proposed to contribute to the intracellular gating structure. To investigate the role of this motif in the destabilization of the α-helix, both prolines were replaced to promote an α-helix (alanine) or to allow a flexible configuration (glycine). These substitutions were nonfunctional or resulted in drastically altered channel gating, highlighting the importance of these prolines. Combining these mutations with a proline substitution scan demonstrated that proline residues in the midsection of S6 are required for functionality, but not necessarily at the positions conserved throughout evolution. These results indicate that the destabilization or bending of the S6 α-helix caused by the PXP motif apparently creates a flexible “hinge” that allows movement of the lower S6 segment during channel gating and opening.

The human K⁺ channel hKv1.5 is a homologue of the Drosophila Shaker K⁺ channel (1). A functional channel is composed of four individual α-subunits, each containing six membrane-spanning segments (S1–S6) and a pore loop between S5 and S6. The latter contains the GYG motif that is typical for potassium-selective channels (2). The crystal structure of KcsA, a two-transmembrane one-pore K⁺ selective channel, has greatly advanced the understanding of ion permeation through a channel pore (3), providing a detailed picture of the mechanism of potassium conduction (4, 5). The recently obtained crystal structure of KvAP, a bacterial 6TM K⁺ voltage-gated K⁺ channel, showed that the ion permeation pathway formed by the S5 through S6 region in this class of channels is indeed quite similar to that of KcsA (6).

The opening and closing of the ion-conducting pore (the gating process) of voltage-gated channels is triggered by a change in the membrane potential, which causes the voltage sensor of each subunit to reorient (for a review, see Ref. 7). This physical reorientation of the voltage sensors results in the opening or closing of the channel gate. Experimental results from various laboratories support the view that the voltage-sensing domain consists of the S2 through S4 region, with the positively charged S4 as the primary component (7). However, in contrast with a previous hypothesis, the crystal structure of KvAP indicates that the voltage sensor is located at the channel’s outer perimeter and adopts a “paddle” conformation. In contrast to our understanding of the voltage sensor, we know considerably less about the molecular nature of the channel gate, and its mechanism of opening and closing remains unclear. Most information has been obtained from the use of channel blockers or cysteine-modifying chemicals (8–10), but it remained unclear whether the gate governing access to these blockers and chemicals was also the gate for K⁺ ions. The effects of small blockers such as Cd²⁺ and Ag⁺ suggested that the activation gate of Shaker is located around valine 478 (Val514 in hKv1.5) in S6 (11, 12). An alanine, valine, and tryptophan substitution scan of the lower part of S6 identified both Val778 and Phe618 (Val514 and Phe517 in hKv1.5) as the residues that most likely occlude the channel pore in the closed state (13).

The sequence alignment of the Kv family reveals a highly conserved proline-X-proline motif (PXP, where X represents a hydrophobic residue) in the lower part of S6 (Fig. 1). Within the Kv family, this motif is fully conserved in members that form functional channels in a homotetrameric configuration. Prolines tend to destabilize (kink) α-helices by the lack of a backbone hydrogen bond (normally formed by the amide nitrogen) and by steric constraints (14–17). Therefore, the structure of the lower part of S6 of eucaryotic Kv channels should be different from that of the bacterial KvAP channel (18); the highly conserved double proline motif may kink the S6 α-helices of eucaryotic Kv channels, resulting in a “bent S6” model as proposed by del Camino et al. (19). Since KvAP lacks this tandem proline motif (Fig. 1), its crystal structure does not resolve the structural consequences of a PXP motif and the possibility of a kink in Shaker-type α-subunits.

The extrapolation of the predicted gating mechanism for KvAP, KcsA, and MthK to Shaker channels would suggest that glycine 466 in Shaker (Gly502 in hKv1.5) acts as a potential hinge or pivoting point (6, 20–22). However, this residue is not conserved within the Kir family, and based on the recent crystal structure of KirBac1.1 (23), it was proposed that Gly142 (the equivalent of Pro475 in Shaker) was the pivoting point for gating, whereas Gly134 (equivalent of Shaker Gly466) was more important for packing. This hypothesis is strengthened, since Gly466 is located four helical turns upstream of the site of the proposed bend and the residues Val778 and Phe618, which are considered to occlude the pore in the closed state. As a consequence, it is still unclear how Shaker-type channels gate and to what extent the conserved PXP motif (Pro473 and Pro475 in Shaker) contributes to this process. We substituted both pro-
Eagle extracellular solution. The cells were perfused continuously with a bath solution containing 110 mM KCl, 5 mM MgCl2, 10 mM Hepes, and adjusted to pH 7.4 with KOH. Junction potentials were zeroed with the filled pipette in the bath solution. The resistance of the patch pipettes was estimated to be 1.7 MΩ (24). After achieving a gigahm seal, the whole cell configuration was obtained by suction. Capacitive transients were elicited by applying a −10 mV voltage step to determine the capacitive surface area, access, and input resistance. The access resistance varied from 3 to 9 megohms without compensation and was below 3 megohms after whole cell compensation.

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**Fig. 1. Alignment of the S6 segment of Kv channels.** The residue numbering of *Shaker* is represented at the top with the corresponding hKv1.5 numbering in parentheses. The position of the residues relative to the first proline of the native motif is shown at the bottom; this numbering is used throughout. The highly conserved proline motif in the Kv channels is indicated in boldface type. The first proline is absolutely conserved, and the second is conserved only in the Kv channels that form functional homotetramers. For completeness, Kvβ.3 is different at positions −4 and +1, and Kvβ.2 is different at −2 and +3 (compared with Kβ.6 and Kβ.9).

**EXPERIMENTAL PROCEDURES**

**Molecular Biology—**hKv1.5 was expressed using a pBK-CMV expression vector. Mutations were introduced into hKv1.5 with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). After the PCR-based mutagenesis, a PstI-BamH1 fragment containing the mutation was cut out of the PCR-amplified vector and ligated in hKv1.5/pBK-CMV to replace the wild-type sequence. Double-stranded sequencing of the exchanged fragment and the adjacent sequence confirmed the presence of the desired modification and the absence of unwanted mutations. Plasmid DNA for mammalian expression was obtained by amplification in XLI2 BlueScript cells (Stratagene). The plasmid DNA was isolated from the bacterial cells with the endotoxin-free Maxiprep kit (Qiagen) and the cDNA concentration was determined with UV absorption.

**Electrophysiology—**Ltk− cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 1% penicillin/streptomycin. The cells were transfected with 0.5–5 μg of cDNA for WT or mutant subunits, following the lipofection method using LipofectAMINE (Invitrogen). 8–24 h after transfection, the cells were trypsinized and used for analysis within 12 h.

Current recordings were made with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) in the whole cell configuration of the patch clamp technique. Experiments were done at room temperature (20–23°C); current recordings were low pass-filtered and sampled at 2–10 kHz with a Digidata 1200A data acquisition system (Axon Instruments). Command voltages and data storage were controlled with pClamp7 software (Axon Instruments). Command voltages and data storage were controlled with pClamp7 software (Axon Instruments, Foster City, CA) in the whole cell configuration of the patch clamp technique.

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The holding potential was −80 mV unless otherwise specified. The interpulse interval was at least 15 s but was increased up to 45 s for some mutants to prevent accumulation of slow inactivation. Details of voltage protocols (voltage range and step duration) were adjusted based upon the different biophysical properties of mutant channels. Time constants of activation and deactivation were determined by fitting the current recordings with a single or double exponential function. The necessity of a second exponential component was judged both by inspection of difference plots and by F-statistics. The voltage dependence of channel activation was fitted with a Boltzmann equation: y = A/(1 + exp(−(E − V)/k)), in which k represents the slope factor, E is the applied voltage, and V is the voltage at which 50% of the channels are activated. Results are expressed as mean ± S.E. Detailed kinetic information of all functional mutants is provided in the Supplemental Material.

**Confocal Imaging—**WT hKv1.5 and nonfunctional proline mutants were tagged with GFP at their carboxyl termini. HEK293 cells in minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% nonessential amino acids were cultured on coverslips and transfected using the lipofection method, with 0.5 μg of channel DNA. Cycloheximide (7.5 μg/ml of medium) was added to the culture medium 18 h after transfection. Another 18 h later, confocal images were obtained on a Zeiss LSM 510, equipped with an argon-ion laser (excitation, 488 nm) for the visualization of GFP-labeled channels.

**Molecular Models—**Molecular models were based upon the known three-dimensional crystal structure of KcsA (3). Within this structure, specific residues were substituted by a proline using the publicly available program Swiss-Pdb Viewer (available on the World Wide Web at expasy.org/spdbv/text/download.htm). Using the Swiss-Pdb viewer, we also altered the torsion angles of the substituted residues, and the proline residues were given their typical φ-ψ bond angles for an α-helical context. These KcsA-based models were subsequently viewed with the program RasMol version 2.5 (available on the World Wide Web at openrasmol.org). The latter program was also used to display the molecular models in Fig. 7.

**RESULTS**

It has been suggested previously that the prolines of the conserved PXP motif cause a kink in the lower part of S6, based on the specific conformation of a proline in an α-helix. This kink would be located near the narrow part of the putative intracellular activation gate (12, 13, 19). To investigate the role of these prolines in channel gating, we mutated both residues to alanine (to force an α-helical configuration) or to glycine, which tends to destabilize an α-helix and could adopt the proline configuration (14, 16). It was suggested that 1) the gate is located in the lower part of S6 and that 2) disruption of the S6 α-helix is essential for a functional gate. We hypothesized that if the proline motif destabilizes the S6 α-helix, the effects of the glycine substitutions would be milder compared with the α-helix-promoting alanine substitutions.

Substitution of the second proline (at position 511 in hKv1.5) with an alanine or a glycine resulted in voltage-dependent K+ currents. The gating kinetics were moderately (P511G) and drastically (P511A) modified (Fig. 2). For the P511A mutation, the midpoint of activation was shifted by at least 100 mV, and the voltage sensitivity (slope factor) was decreased by a factor of 2 compared with WT hKv1.5 (Fig. 3A and Table 1). The time constants of activation and deactivation were extremely slow, −300 times slower than WT (Fig. 3B). In contrast, the overall effects of a glycine at the same position (P511G) were milder than those of the alanine mutation (P511A). Compared with WT hKv1.5, the voltage dependence of activation of P511G was shifted by about 60 mV in the positive direction (Fig. 3A) with time constants that were only 10 times slower (Fig. 3B and Table 1).

In contrast, substitution of the first proline by an alanine or glycine resulted in voltage-dependent K+ currents. The gating kinetics were moderately (P511G) and drastically (P511A) modified (Fig. 2). For the P511A mutation, the midpoint of activation was shifted by at least 100 mV, and the voltage sensitivity (slope factor) was decreased by a factor of 2 compared with WT hKv1.5 (Fig. 3A and Table 1). The time constants of activation and deactivation were extremely slow, −300 times slower than WT (Fig. 3B). In contrast, the overall effects of a glycine at the same position (P511G) were milder than those of the alanine mutation (P511A). Compared with WT hKv1.5, the voltage dependence of activation of P511G was shifted by about 60 mV in the positive direction (Fig. 3A) with time constants that were only 10 times slower (Fig. 3B and Table 1).
If these proline residues disrupt the function of P511A were extremely slow (note positive voltages were needed to obtain full and longer depolarizing steps to more positive voltages were needed to obtain full activation. The activation and deactivation of P511A were extremely slow (note the difference in scale bars and pulse protocols). Notice that 5-s depolarizing steps to voltages up to +120 mV were insufficient to reach full saturation of the tail currents obtained at -20 mV.

glycine (P509A and P509G) was not tolerated, since no time-dependent current was observed within a voltage range of -130 to +130 mV, even with 5- or 10-s voltage steps. The double mutant P509A/P511A, which should result in an α-helical conformation of the lower part of S6, comparable with KvAP and KcsA, was nonfunctional too. Confocal imaging showed that the nonfunctional proline mutants were properly expressed at the plasma membrane as shown by the prominent GFP staining (Fig. 4).

The results indicate that the PXP motif plays an essential role in gating and that there appears to be an absolute requirement for the presence of the first proline. This is consistent with previous suggestions of their role in a kinked S6 α-helix. If these proline residues disrupt the α-helical structure of S6 to create a specific conformation, we reasoned that the introduction of a proline on a position other than 509 or 511 might rescue the functionality of the P509A/G, P509A/P511A, or P511A/G mutations. To explore this possibility, we performed a proline-scanning mutagenesis in these different backgrounds (Table I).

**Rescue of P509G**—Remarkably, the nonfunctional P509G channel was rescued by the introduction of a proline at positions -3, -2, and +3 (the numbering is given with respect to the position of the first proline of the native motif, which is indicated in boldface type (i.e. -2P in P509G corresponds to PXGXP; see also Table I). This indicates that quasinormal voltage-dependent gating is possible with proline residues at positions that differ from the evolutionarily conserved ones.

The voltage-dependent kinetics of the rescue mutant -2P in P509G (i.e. PXGXP) were comparable with WT (Table I; detailed kinetic information of all mutations is available in the Supplemental Material). In all cases, a control mutation was inserted by inserting the specified proline in a WT subunit. In the case of -2P in P509G, a proline was inserted at position -2P in a WT subunit, thus creating PXPPX. The control mutation had biophysical properties that were similar to those of -2P in P509G. This similarity suggested that the configuration of the glycine 509 in a PXGXP motif mimics that of a proline in a PXPPXP motif. The biophysical properties of the rescue mutant -3P in P509G (i.e. PXGXP) were comparable with WT (Table I). The +3P rescue mutant (i.e. GXPXP) had a voltage dependence of channel activation comparable with that of the +3P mutation in WT (i.e. PXXP) (Table I), but the kinetic properties were clearly different. The activation and deactivation time course was monoeponential in the control mutation (+3P in WT) but was composed of two components in the case of +3P in P509G. The difference between +3P in P509G and in WT suggested that, in contrast with a PXGXP motif mentioned earlier, the glycine 509 in a GXPXP motif adopted a conformation different from that of the corresponding proline in PXPP.

None of the other proline mutants (Table I) could rescue the P509G mutation, as judged by the lack of time-dependent K⁺ current within the voltage range of -130 to +130 mV. Taken together, these results indicated that the presence of proline residues was essential, but that prolines at positions -3, -2, and +3 relative to Pro509G were tolerated in addition to the evolutionarily conserved ones.

**Rescue of P509A and P509A/P511A**—Introduction of a proline at position -2, -1, or +3 in the nonfunctional P509A and P509A/P511A mutant backgrounds resulted in subunits that displayed time-dependent currents (Table I). The kinetics of the -2P and -1P rescue mutants in the P509A mutation (i.e. PXAXA and PAXA) approximated the WT kinetics. The double mutant P509A/P511A was also rescued by the -2P and -1P mutations (i.e. PXAXA and PAXA) (Fig. 5, A and B, and Table I). However, the rescue mutations in P509A compared better with WT than these in P509A/P511A, possibly because the extra proline (Pro511G) was still present in P509A. Remarkably, the -1P mutation did not rescue P509G and also conferred nonfunctionality to WT (Table I). The +3P mutation rescued both P509A and P509A/P511A (i.e. AXPP and AXAP) (Fig. 6A). However, in contrast to the -2P and -1P rescue mutations, the time course of activation was extremely slow (>100-fold slower than WT) and even displayed an inverse voltage de-
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Fig. 3. Biophysical properties of WT hKv1.5 (triangles), P511G (open circles), and P511A (filled circles) mutants. A, voltage dependence of activation: “isochronal” $P_o$ values, derived from the tail currents in Fig. 2, were plotted as a function of the prepulse potential. Compared with WT hKv1.5, the activation curve of P511G and P511A was shifted toward positive voltages. Note that in contrast with WT and P511G, the activation of P511A did not reach full saturation. B, gating kinetics. Time constants derived from monoeXponential fits of the raw current traces (Fig. 2) were plotted as a function of membrane potential. Both the activation and deactivation time constants of P511G were extremely slow (note the semilogarithmic axis).

The P511G activation of P511A did not reach full saturation. The mid-point potentials were at least positive to 50 mV with slope factors over 20 mV (Fig. 6B). Furthermore, 5-s voltage steps to +90 mV failed to activate the channels completely (Fig. 6A). Thus, the activation curves did not reach full saturation, and the mid-point potentials were at least positive to +50 mV with slope factors over 20 mV (Fig. 6B).

Interestingly, these results did show that the nonfunctional P509A/P511A channel (i.e. with an AXA motif instead of PXP) could be rescued by the introduction of a single proline at another position in the lower part of S6. Moreover, the introduction of a proline at position −2 or −1 (i.e. PXAXA and PAXA) was better tolerated than at +3 (i.e. AXAP) (i.e. biophysical properties of these mutants were much closer to WT). The latter effect was also observed in the P509A and P509G background. To explore the importance of the relative position of the two proline residues of the native motif, we inserted two prolines at nonevolutionarily conserved positions in an $\alpha$-helix (Pro509 and Pro511) of the native motif by an alanine or a glycine was not tolerated. The former two prolines at nonevolutionarily conserved positions in an $\alpha$-helix suggest at least a 20° bend in the axis of the $\alpha$-helix. Consistent with this, cysteine accessibility studies suggested a “bent S6” model for Shaker-type channels (19) (i.e. an S6 that differs from the linear $\alpha$-helix of KcsA and KvAP) (3, 6). Also, on the basis of molecular modeling, the PXP motif was proposed to form a flexible “hinge” structure (25, 26). It was proposed that this “bend” or “hinge” is important for functional gating in this class of Kv channels (12). Indeed, substituting both proline residues of the PXAXAP motif with alanine (P509A/P511A) resulted in a nonfunctional channel. Even substituting the first proline (Pro509) of the motif by an alanine or a glycine was not tolerated, similar to the P473A substitution in Shaker (13). Mutating the second proline (Pro511) resulted in channels with clearly disrupted channel gating, whereas P475A was reported to be nonfunctional in Shaker (13). The nonfunctionality of P509A/G and of the double P509A/P511A mutant was not caused by folding or trafficking problems, since proper expression at the plasma membrane was shown by confocal microscopy. Thus,

The activation and deactivation time constants were extremely slow (note the semilogarithmic axis). Note that in contrast with WT and P511G, the activation of P511A did not reach full saturation.
the nonfunctional proline mutants and the profound effects of the P511A/G mutations can be explained by a channel with a disrupted intracellular activation gate (e.g. one that is constantly closed and/or disjoined from the activation machinery). For the nonfunctional P473A and P475A mutation in Shaker, it was suggested that trapping occurred in the inactivated state (13). Our data do not support this hypothesis, because P511A was functional, and the activation curve of P511A (forcing an S6 helix) was shifted more positive compared with P511G. The mutant P509A/P511A should result in a straight lower S6 helix (similar to KvAP and KcsA). This mutant did not display voltage-dependent currents, although the channel was expressed at the cell surface. The nonfunctional alanine and glycine mutations could be rescued by the introduction of a proline upstream or downstream of Pro509. The positions 2, 1, or 3 (relative to Pro 509) are crucial for rescuing. These results confirm that the PXP motif or a similar flexibility by different proline residues in the lower part of S6 is important for channel gating. Furthermore, the results obtained with the mutant P509A/P511A indicate that great caution is needed with extrapolations from the crystal structure in terms of channel gating, although the interpretations of ion permeation undoubtedly hold.

The PXP Motif Disrupts (Kinks) the S6 α-Helix—Since a glycine has more degrees of freedom and tends to destabilize an

### TABLE I  
**Proline scan of the lower part of S6**

| S6 | I | $V_{1/2}$ (mV) | k | $\tau$ (ms) | n |
|----|---|----------------|---|------------|---|
| WT hKv1.5 | T I A L P V P V I V | +++ | -14.3 ± 0.3 | 6.3 ± 0.1 | 3 ± 0.3 | 5 |
| P509G | T I A L Q V P V I V | - | - | - | - | - |
| P509A | T I A L A V P V I V | - | - | - | - | - |
| P511G | T I A L P V G V I V | +++ | 44 ± 1 | 8.6 ± 0.2 | 36 ± 2 | 10 |
| P511A | T I A L P V A V I V | +++ | 90 ± 2 | 15.6 ± 0.4 | 1240 ± 180 (at +120 mV) | 7 |
| P509A/P511A | T I A L A V A V I V | - | - | - | - | - |
| -4P in P509G | P I A L G V P V I V | + | -28 ± 2.6 | 6.0 ± 0.4 | 4.8 ± 0.5 | 5 |
| -3P | T P A L G V P V I V | + | -32 ± 2 | 8.4 ± 0.6 | 5.8 ± 0.8 | 4 |
| -2P | T I A L G P V P I V | - | - | - | - | - |
| -1P | T I A L G V P P I V | - | - | - | - | - |
| +1P | T I A L G V P P I V | - | - | - | - | - |
| +3P | T I A L G V P P I V | +++ | -30 ± 1 | 9.5 ± 0.5 | 21 ± 4.3 | 185 ± 33 | 5 |
| +4P | T I A L G V P P I V | - | - | - | - | - |
| -2P in P509A | T I P A L V P I V | + | -21.9 ± 1.8 | 5.4 ± 0.3 | 3.7 ± 0.4 | 4 |
| -1P | T I A P A V P I V | + | -20 ± 1.2 | 7.6 ± 0.9 | 3.7 ± 0.4 | 7 |
| +3P | T I A L A V P P I V | +++ | ND | ND | ND | 5 |
| +4P | T I A L A V P P I V | - | - | - | - | - |
| -2P in P511G | T I P A L P G V I V | +++ | -53 ± 1 | 5.5 ± 0.3 | 6 ± 0.5 | 6 |
| -1P | T I A P A P G V I V | + | -14 ± 1.1 | 6.2 ± 0.3 | 3.5 ± 0.3 | 8 |
| +1P | T I A L P G P V I V | - | - | - | - | - |
| +3P | T I A L P G P V I V | +++ | -7.3 ± 1.3 | 6.2 ± 0.2 | 49 ± 6 | 7 |
| +4P | T I A L P G P V I V | - | - | - | - | - |
| -2P in P511A | T I P A L V A V I V | +++ | -5 ± 2 | 6.8 ± 0.3 | 7 ± 1 | 5 |
| -1P | T I A P A V A V I V | +++ | 5.3 ± 0.8 | 5.7 ± 0.4 | 8.2 ± 0.2 | 8 |
| +3P | T I A L A V P A V I V | +++ | 8 ± 0.8 | 6 ± 0.3 | 26 ± 3 | 183 ± 23 | 8 |
| +4P | T I A L A V P A V I V | - | - | - | - | - |
| -2P in WT hKv1.5 | T I P A L P V P I V | + | -31 ± 2 | 5.5 ± 0.7 | 4.3 ± 0.6 | 7 |
| +3P | T I A P A P P V I V | - | - | - | - | - |
| -2P in P509A/P511A | T I P A L A V A V I V | +++ | 15 ± 1.2 | 7.1 ± 0.6 | 69 ± 4 (at +90 mV) | 4 |
| +3P | T I A L A V P A V I V | +++ | 5 ± 1.7 | 10 ± 0.6 | 9 ± 0.7 | 10 |
| +4P | T I A L A V P A V I V | - | ND | ND | ND | 6 |
| -2P+3P | T I P A L A V A V I V | +++ | -25 ± 1.2 | 6.4 ± 0.4 | 130 ± 16/1300 ± 230 | 10 |

### FIG. 4. Subcellular localization of WT hKv1.5 and P509A/P511A GFP fusion proteins by confocal imaging. A, hKv1.5 GFP fusion proteins displayed prominent green fluorescent staining at the level of the plasma membrane. The scale bar indicates 10 μm. B, the nonfunctional proline mutant P509A/P511A displayed a similar plasma membrane staining, demonstrating proper expression at the plasma membrane.
α-helix like a proline residue, we expected that if the prolines disrupt (kink) the α-helical configuration of S6, the effects of glycine mutations would be milder compared with the α-helix-promoting alanine mutations. This hypothesis is supported by the observations that 1) the gating parameters of P511A were more drastically altered compared with these of P511G, and 2) the biophysical parameters of the +3P rescue mutation in P509A (i.e., GXPP) compared better with the control mutation than those in P509A. The fact that the nonfunctional glycine and alanine mutants could be rescued by the introduction of a proline in this region (between positions 506 and 513) indicates that the prolines destabilize and disrupt the lower part of the S6 α-helix. Surprisingly, for channel functionality, the prolines are needed but not at the evolutionary conserved position, as

![Current recordings of several rescue mutations](image)

**Fig. 5.** Current recordings of several rescue mutations. The maximum applied voltage was always +60 mV and corresponded with the top current trace. The horizontal bar on the left indicates the zero current level. The nonfunctional P509A and P509A/P511A were clearly rescued by the introduction of a proline at position −1 (A and B). The kinetics of the −1P rescue mutations were similar to WT. The threshold for activation of −1P in P509A was at more negative voltages when compared with −1P in P509A/P511A and was associated with a slightly faster activation. C, the mutant −2P+3P in P509A/P511A with a PXAXAP sequence. Remarkably, this mutant channel was functional, albeit with gating kinetics clearly different from WT. D and E, −2P and −1P in P511A. Compared with P511A, the introduction of a proline at positions −1 and −2 resulted in channels with currents comparable with WT. F, +3P in P511A. The activation reached saturation, and time constants were clearly faster than P511A. Compare the biophysical properties of D, E, and F with the placement of the introduced proline. Notice that a PXPA sequence, with the introduced proline upstream of Pro509, approximated WT better than a PXAP motif (F), in which the proline is introduced downstream of Pro509.

![Biophysical properties of +3P in P509A (open circles) and P509A/P511A (filled circles)](image)

**Fig. 6.** Biophysical properties of +3P in P509A (open circles) and P509A/P511A (filled circles). A, activation determined with a voltage protocol as shown at the top. Currents obtained for +3P in P509A (middle panel) and +3P in P509A/P511A (lower panel) indicated that both proline mutations clearly rescued the nonfunctional alanine mutants. However, both rescue mutations resulted in channels with drastically altered gating kinetics (i.e., both mutants activated extremely slowly). B, voltage dependence of activation. The normalized tail current amplitudes (from A) were plotted as a function of the prepulse potential. The WT hKv1.5 activation curve is represented with a dotted line. Note that both the activation curves of +3P in P509A (AXPP) (n = 5) and P509A/P511A (AXAP) (n = 6) did not saturate, indicating that activation was not complete within this voltage range. C, gating kinetics. Time constants of activation, derived from monoeXponential fits of the raw current traces, were plotted as a function of potential. Both mutants were extremely slow (note the semilogarithmic axis). Also, the voltage dependence was inverted as time constants became slower at more depolarized potentials.
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Fig. 7. Models of KcsA (AXA), −2P in KcsA (PXAXA), 0P in KcsA (PX). The top panel shows a top view of the complete tetrameric channel. Below is a side view of one α-subunit (representing S6 through S6). In B and C, one subunit is removed in the top view to better illustrate the relative orientation of the lower part of S6. Blue, S5; red, S6; orange, P-loop; yellow, GYG; green, residue +5V (residue Val578 in Shaker), white, proline residue at position −2 (Pro507) and 0 (Pro509) in B and C, respectively. A, the crystal structure of KcsA (AXA). No proline is present, and S6 forms a straight α-helix. Starting from the KcsA structure, a proline residue is introduced on position −2 (PXAXA) (B) and 0 (PXAXA) (C). In B and C, the proline was given torsion angles of a trans arrangement in an α-helical context (ψ = −70°, −35°) with the repercussions of a curved α-helix (15). Note that in contrast with KcsA, the lower part of S6 is curved and that the orientation is the opposite in both B and C. However, both channels −2P in P509A/P511A (PXAXA) (B) and P511A (PXAXA) (C) were functional with similar properties. This intuitively shows that both must have a common orientation.

was illustrated by the various proline rescue mutations. This indicates that it is not the position of the proline that is essential but the destabilization of the α-helical configuration of S6.

Proline residues in an α-helical configuration result, due to their typical ψ–φ bond angles, in a curved or kinked α-helix. We propose that the prolines not only curve the α-helix but kink it, since a curved α-helix would result in different S6 orientations between several functional proline mutants (Fig. 7). However, functionality most likely requires a similar well defined orientation of the lower S6 α-helix. In a kinked α-helix, the kink and the residues after the kink can orient the lower part of S6 in all mutants in a similar direction, which is essential for functional gating (e.g. for creating the putative S4-gate link). The mutant with a PXAXA sequence supports this idea, since it has nearly WT kinetics, although in this configuration the position of the first proline of the motif is the opposite compared to WT, when represented on an α-helical wheel. Also when the “single” proline mutants P511A (PXAXA) and −2P in P509A/P511A (PXAXA) are compared, the position of the proline is the opposite. As such, the comparison of structural elements in different “single” proline mutations can lead to a common orientation of the lower part of S6 in functional channels. Within this view, these proline mutations might be beneficial for molecular modeling (25).

The Residues after the First Proline Create a “Hinge” Region or Pivoting Point—Since a proline disrupts (kinks) the S6 α-helix, the residues located downstream of this disruption have a greater degree of freedom, suggesting a flexible structure. As a consequence, the position of the first proline is of importance. Indeed, the rescue mutation with a PXAXA motif (−2P in P509A) had biophysical properties that compared well with the control mutation −2P in WT hKv1.5 (i.e. a PXAXA motif). On the other hand, the +3P rescue mutation with an AXAP motif (+3P in P509A) did not compare with the control mutation in WT (i.e. a PXPP motif). The difference between the rescue mutations mentioned above is the position of the first proline (kink) with respect to position 509 (i.e. upstream or downstream). This was observed in all mutant backgrounds, since the −2P and −1P were better rescue mutations than the +3P. These observations indicate, as suggested previously (25, 26), that the proline residues and the residues between them form a flexible “hinge” region, which may serve as a pivoting point during channel opening and closing. Probably, two proline residues can form a better “hinge,” since the proline rescue mutations of P509A compared more with WT than those of P509A/P511A. Also, the rescue of the biophysical properties of the P511G and P511A mutant by the introduction of a second proline can be explained by an increased flexibility of the lower part of S6. Apparently, channels may form a long flexible “hinge” region (e.g. a PXAXA (Fig. 5C) and a PXAXGP (−3P in P509G) sequence) and still be functional. These data support and reinforce the idea that a “bend/hinge” structure is required for the conformational change of the intracellular activation gate during channel opening and closing, consistent with results from state-dependent blocker protection (12, 19). As a consequence, our data also indicate that the intracellular gate for channel blockers and cysteine-modifying chemicals is also the site for gating K+ movements.

The comparison of the crystal structures of KcsA and MthK suggested that the pivoting point for gating is formed by a highly conserved glycine corresponding to Gly166 in Shaker (Gly562 in hKv1.5) (20–22). The recent crystal structure of KvAP supports this model, since there is a clear bending of S6 at this conserved glycine (6). A proline substitution scan study in GIRK4 channels, G-protein-sensitive K+ channels, demonstrated that introduced prolines disrupt and kink the second transmembrane (TM2) helix of these channels (27). The authors concluded that the gating of this type of channel also depends on this highly conserved glycine present in GIRK4. However, KvAP and these other channels lack a tandem proline motif (Fig. 1), and our data clearly show that the PXP motif is absolutely required for gating. Furthermore, from the crystal structure of KirBac1.1 (23) a glycine (Gly134) that is the equivalent of the second proline of the PXP motif in Shaker is interpreted as pivoting point for gating of this class of channels, whereas the glycine Gly134 in KirBac1.1 (corresponding to Gly166 in Shaker) is considered to be restricted and involved in packing. Whereas we cannot exclude the possibility that Gly166 in Shaker is flexible as in KvAP, KcsA, MthK, and GIRK4 (20–22, 27), our results suggest that the prolines form either the main “hinge” or pivoting point for voltage-dependent gating or create a second one on top of the conserved glycine.

In conclusion, our results indicate that in Shaker-type K+ channels, the lower part of S6 contributes to a functional intracellular activation gate, which reorients during channel opening and closing. The conformational change of the gate requires a disrupted (kinked) S6 α-helix caused by the evolutionarily conserved PXP motif, creating a flexible “hinge” region. Furthermore, our results demonstrate that for functionality, the position of these proline residues is not necessarily the evolutionarily conserved one.

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REFERENCES
1. Snyders, D. J., Tamkun, M. M., and Bennett, P. B. (1993) J. Gen. Physiol. 101, 513–543
2. Heginbotham, L., Lu, Z., Abramson, T., and MacKinnon, R. (1994) Biophys. J. 66, 1061–1067
3. Doyle, D. A., Cabral, J. M., Pfeutzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
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4. Morais-Cabral, J. H., Zhou, Y., and MacKinnon, R. (2001) Nature 414, 37–42
5. Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Nature 414, 43–48
6. Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T., and MacKinnon, R. (2003) Nature 425, 33–41
7. Bezanilla, F. (2000) Physiol. Rev. 80, 555–592
8. Armstrong, C. M. (1971) J. Gen. Physiol. 58, 413–437
9. Choi, K. L., Mossman, C., Aube, J., and Yellen, G. (1993) Neuron 10, 533–541
10. Holmgren, M., Smith, P. L., and Yellen, G. (1997) J. Gen. Physiol. 109, 527–535
11. Liu, Y., Holmgren, M., Jurman, M. E., and Yellen, G. (1997) Neuron 19, 175–184
12. del Camino, D., and Yellen, G. (2001) Neuron 32, 649–656
13. Hackos, D. H., Chang, T. H., and Swartz, K. J. (2002) J. Gen. Physiol. 119, 521–532
14. Blaber, M., Zhang, X. J., and Matthews, B. W. (1993) Science 260, 1637–1640
15. MacArthur, M. W., and Thornton, J. M. (1991) J. Mol. Biol. 218, 397–412
16. O’Neil, K. T., and DeGrado, W. F. (1990) Science 250, 646–651
17. von Heijne, G. (1991) J. Mol. Biol. 218, 499–503
18. Barlow, D. J., and Thornton, J. M. (1988) J. Mol. Biol. 201, 601–619
19. del Camino, D., Holmgren, M., Liu, Y., and Yellen, G. (2000) Nature 403, 321–325
20. Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 417, 523–526
21. Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 417, 515–522
22. Yifrach, O., and MacKinnon, R. (2002) Cell 111, 231–239
23. Gus, A., Gultis, J. M., Astleff, J. F., Rahman, T., Lowe, E. D., Zimmer, J., Cuthbertson, J., Ashcroft, F. M., Ezaki, T., and Doyle, D. A. (2003) Science 300, 1922–1926
24. Neher, E. (1992) Methods Enzymol. 207, 123–131
25. Bright, J. N., Shrivastava, I. H., Cordes, F. S., and Sansom, M. S. (2002) Biopolymers 64, 303–313
26. Tieleman, D. P., Shrivastava, I. H., Ulmschneider, M. R., and Sansom, M. S. (2001) Proteins 44, 63–72
27. Jin, T., Peng, L., Miremba, T., Rohacs, T., Chan, K. W., Sanchez, R., and Logothetis, D. E. (2002) Mol. Cell 10, 469–481