Published in final edited form as:

Biochemistry. 2006 February 14; 45(6): 1663–1672. doi:10.1021/bi0516430.

NMR-derived dynamic aspects of N-type inactivation of a Kv channel suggest a transient interaction with the T1 domain

Kent A. Baker¹, Christian Hilty^{2,*}, Wolfgang Peti^{3,§}, Alison Prince⁴, Paul J. Pfaffinger⁴, Gerhard Wider², Kurt Wüthrich^{2,3}, and Senyon Choe^{1,5}

¹Structural Biology Laboratory, The Salk Institute, La Jolla, CA 92037, USA ²Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, 8093 Zürich, Switzerland ³The Scripps Research Institute, La Jolla, CA 92037, USA ⁴Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA

Abstract

Some eukaryotic Kv channels contain an N-terminal inactivation peptide (IP), which mediates a fast inactivation process that limits channel function during membrane depolarization and thus shapes the action potential. We obtained sequence-specific NMR assignments for the polypeptide backbone of a tetrameric N-terminal fragment (amino acids 1–181) of the *Aplysia* Kv1.1 channel. Additional NMR measurements show that the T1 domain has the same globular structure in solution as previously determined by crystallography, and that the IP (residues 1–20) and the Linker (residues 21–65) are in a flexibly disordered, predominantly extended conformation. A potential contact site between the T1 domain and the flexible tail (residues 1–65) has been identified based upon chemical shift changes of individual T1 domain amino acids, which maps to the T1 surface near the interface between adjacent subunits. Paramagnetic perturbation experiments further indicate that in the ensemble of solution conformers, there is at least a small population of species with the IP localized in close proximity to the proposed interacting residues of the T1 tetramer. Electrophysiological measurements show that all three mutations in this pocket we tested slow the rate of inactivation and speed up recovery, as predicted from the pre-inactivation site model. These results suggest that specific, short-lived transient interactions between the T1 domain and the IP or the Linker segment may play a role in defining the regulatory kinetics of fast channel inactivation.

Cytoplasmic domains of voltage-gated K⁺ (Kv) channels regulate channel assembly, trafficking and function, and control cytoplasmic access to the ion conduction pathway (1–7). Inactivation of Kv channels is a critical biological timing mechanism that limits the duration of channel activity in response to depolarization, as well as suppressing further channel activity for a period of time following depolarization. Thus, the ability to inactivate is an essential property of a variety of channels involved in shaping and regulating the excitability of the cell (2). A particular form of rapid inactivation of Kv channels, called N-type inactivation, is mediated by an inactivation peptide (IP) segment of approximately 20 amino acids located at the N-terminal end of the α -subunits or of certain associating β -subunits. Results from experiments with free synthetic IP's, which restore inactivation to IP-free Kv channels (3,8,9), support the idea that a single IP can function by physically

⁵For correspondence: choe@salk.edu, phone (858)453-4100, fax (858) 452-3683.

*Present address: Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.

§Present address: Brown Medical School, Providence, RI 02912, USA.

occluding the central ion pathway of open channels (10,11), independent of its covalent association with the channel.

Because of the presence of the bulky tetrameric T1 assembly located between the IP and the central IP-binding site (12–14), and since the central cavity of the T1 domain is not part of the ion conduction pathway (15), it has been hypothesized that there must exist a pathway that allows the IP to move around the intervening T1 domain to reach its target site within the transmembrane (TM) pore of the channel, and that the lateral “side-windows” formed between T1 and the TM domains most likely provide this route (14–18). This structural framework was derived from the recent crystal structure of rat Kv1.2 in complex with K β 2 associated with the N-terminal membrane-distant side of the T1 domain (19). Given the narrowness of the lateral openings in the structure, it appears that the IP and the Linker segment would have to be highly flexible, non-globular structures to access the ion conduction pathway of the TM pore domain.

NMR structures of isolated IP's display different protein conformations, from unstructured to ball-shaped (20,21). These observations indicate that the IP could either always be disordered and available to bind inside the channel after the pore opens, or that the IP and the Linker interact with other parts of the channel until they are triggered to unfold. If the IP is always ready to bind, then the rate of inactivation will be largely driven by the availability of the binding site. Alternatively, if the IP and the Linker remain preferentially bound to their residential ‘pocket’, they may need to disengage and/or unfold to bind to their IP-binding site. In order to address the structural framework of the IP and the Linker segment in relation to the T1 domain, we have carried out a NMR investigation of the N-terminal domain of the *Aplysia* Kv1.1 voltage gated potassium channel, aKv1.1N, which contains residues 1 to 181 (Figure 1), ending approximately 5 amino acids prior to the first TM helix, S1 (19). The aKv1.1N protein is a soluble tetrameric assembly with a molecular weight of approximately 84 kD. TROSY-based NMR methods (22) on high field instruments, and the use of uniform and amino acid-specific stable-isotope labeling allowed for the sequence-specific resonance assignment as the basis for structural characterization. Additionally, the resonance assignment of the T1 domain tetramer of residues 66-173, aKv1.1T1, was also completed. Finally, paramagnetic probes attached at membrane-proximal, central or membrane-distant regions of the T1 domain were used to estimate the extent of transient proximity of the IP and the Linker with respect to different surface areas of the T1 domain.

MATERIALS AND METHODS

Protein preparation

Two expression constructs were used based on the Shaker Kv1.1 gene from *Aplysia* (M95914). The first construct used the coding sequence for amino acids 66-173 of the gene in the vector pET-24a and included a non-cleavable C-terminal 6xHis tag (13). The second construct used the coding sequence for amino acids 1-181 in a customized pET-28 and included a thrombin cleavable N-terminal 8xHis tag leaving four additional amino acids (Gly-Ser-His-Gly) attached at the N-terminus after cleavage. Four amino acid substitutions were introduced in the native sequence of (R30A, R38A, C63S, C65S) aKv1.1N to help improve protein stability. These mutations, when introduced into the intact channel, do not affect the inactivation properties of the intact channel. Proteins were expressed in BL-21(DE3) *E. coli* and purified in a single step by metal affinity chromatography.

Two expression methods were used to produce uniformly isotope labeled protein. The first is an adaptation of the method published by Marley et al. (23). BL21(DE3) *E. coli* were transformed with the desired expression construct, grown at 37°C in LB to O.D.(600 nm) $\approx$ 0.7 then spun down, rinsed with PBS and resuspended in minimal media containing the

isotope label. A volume ratio of 4:1 LB to minimal media was used to produce a high-density sample prior to induction. After 1 hour, bacteria were induced with 0.5 mM IPTG for 5 hours at 37°C. This method allowed for high levels of protein expression but reduced isotope incorporation to approximately 80%. Proteins with ^{15}N selectively labeled amino acids were produced using the auxotrophic DL-39 *E. coli* strain (24). Expression was similar to the above but with defined media containing a single ^{15}N labeled amino acid replacing the minimal media (25). Samples of aKv1.1N were produced with aspartic acid, leucine, phenylalanine and valine selectively labeled. Uniformly labeled samples were also produced using Celtone isotope labeling media (Spectra Stable Isotopes) in order to increase the level of isotope incorporation. Bacteria were grown at 37°C in Celtone to an O.D. (600) $\approx$ 0.7 then induced with 0.5 mM IPTG for 5 hours.

After growth and induction, the cells were lysed, the resulting lysate was centrifuged to clear cellular debris and applied to metal affinity resin. The resin was thoroughly rinsed, and then the purified protein was eluted with 0.25 M imidazole. Samples with cleavable His tags were treated with 1:1500 mass ratio of thrombin overnight prior to final processing. Purified protein samples were exchanged into 20 mM sodium phosphate pH 7.0, 50 mM NaCl, 5% D_2O and concentrated by ultrafiltration, unless otherwise noted. Sample concentrations ranged from 0.7–1.5 mM monomeric protein.

NMR spectroscopy

A 3D TROSY-HNCA spectrum was recorded with $[70\% ^2\text{H}, \text{u}-^{13}\text{C}, ^{15}\text{N}]$ -aKv1.1T1 on a Bruker Avance 800 spectrometer with 40 transients per increment (26). Time domain data size was $32 (t_1) \times 41 (t_2) \times 512 (t_3)$ complex points, and evolution times $t_{1\text{max}}(^{15}\text{N}) = 13.1$ ms, $t_{2\text{max}}(^{13}\text{C}) = 7.8$ ms, $t_{3\text{max}}(^1\text{H}) = 53.3$ ms were used. Of the same sample, 3D $[^{13}\text{C}]$ -*ct*-TROSY-HNCA (27) and 3D TROSY-HNCACB (28) spectra were recorded on a Bruker Avance 900 spectrometer, the former with time domain data size $32 (t_1) \times 150 (t_2) \times 512 (t_3)$ complex points, evolution times $t_{1\text{max}}(^{15}\text{N}) = 11.0$ ms, $t_{2\text{max}}(^{13}\text{C}) = 23.4$ ms, $t_{3\text{max}}(^1\text{H}) = 40.6$ ms and 16 transients per increment, and the latter with time domain data size $32 (t_1) \times 80 (t_2) \times 1024 (t_3)$ complex points, evolution times $t_{1\text{max}}(^{15}\text{N}) = 12.5$ ms, $t_{2\text{max}}(^{13}\text{C}) = 6.2$ ms, $t_{3\text{max}}(^1\text{H}) = 81.2$ ms and 32 transients per increment. On a 500 MHz spectrometer with a cryogenic probehead, a (H)N(COCA)NH spectrum (29) of $[\text{u}-^2\text{H}, ^{13}\text{C}, ^{15}\text{N}]$ -aKv1.1N was measured with a time domain data size of $35(t_1) \times 64(t_2) \times 512(t_3)$ complex points. Evolution times were $t_{1\text{max}}(^{15}\text{N}) = 21$ ms, $t_{2\text{max}}(^{15}\text{N}) = 39$ ms, $t_{3\text{max}}(^1\text{H}) = 79$ ms, and 8 transients per increment were acquired. All NMR experiments were performed at 37°C. $[^1\text{H}, ^1\text{H}]$ -NOESY- $[^{15}\text{N}, ^1\text{H}]$ -TROSY spectra (30) were recorded of $[70\% ^2\text{H}, \text{u}-^{15}\text{N}]$ -aKv1.1T1 with nuclear Overhauser effect (NOE) mixing times of 60 and 120 ms. The 60 ms spectrum was recorded on a Bruker Avance 800 spectrometer with $150 (t_1) \times 37 (t_2) \times 1024 (t_3)$ complex points, $t_{1\text{max}}(^1\text{H}) = 15.6$ ms, $t_{2\text{max}}(^{15}\text{N}) = 15.2$ ms, $t_{3\text{max}}(^1\text{H}) = 106$ ms and 16 transients per increment. The 120 ms spectrum was recorded on a Bruker Avance 900 spectrometer with $128 (t_1) \times 32 (t_2) \times 1024 (t_3)$ complex points, $t_{1\text{max}}(^1\text{H}) = 11.9$ ms, $t_{2\text{max}}(^{15}\text{N}) = 11.7$ ms, $t_{3\text{max}}(^1\text{H}) = 95.0$ ms and 16 transients per increment. Of $[\text{u}-^2\text{H}, ^{13}\text{C}, ^{15}\text{N}]$ -aKv1.1N, a $[^1\text{H}, ^1\text{H}]$ -NOESY- $[^{15}\text{N}, ^1\text{H}]$ -TROSY spectrum was recorded on a Bruker Avance 900 MHz spectrometer with a NOE mixing time of 200 ms, time domain data size $138 (t_1) \times 35 (t_2) \times 1024 (t_3)$ complex points, $t_{1\text{max}}(^1\text{H}) = 12.8$ ms, $t_{2\text{max}}(^{15}\text{N}) = 12.0$ ms, $t_{3\text{max}}(^1\text{H}) = 97.5$ ms and 16 transients per increment. With the same sample, a 900 MHz 3D TROSY-HNCA experiment was recorded with time domain data size $32 (t_1) \times 32 (t_2) \times 512 (t_3)$ complex points, $t_{1\text{max}}(^{15}\text{N}) = 12.6$ ms, $t_{2\text{max}}(^{13}\text{C}) = 5.0$ ms, $t_{3\text{max}}(^1\text{H}) = 43.9$ ms and 64 transients per increment. The 800 and 900 MHz spectrometers were equipped with three high-power radio-frequency channels for the ^1H , ^{13}C and ^{15}N pulses, low-power 20W amplifiers for ^2H -decoupling, and ^1H - $\{^{13}\text{C}, ^{15}\text{N}\}$ triple resonance probes with actively shielded gradients. TROSY-based $^{15}\text{N}\{^1\text{H}\}$ -NOE experiments (31) were measured of

[70% ^2H , $^{\text{u-}}^{13}\text{C}$, ^{15}N]-aKv1.1T1 and [70% ^2H , $^{\text{u-}}^{13}\text{C}$, ^{15}N]-aKv1.1N on a Bruker DRX 500 spectrometer equipped with four high-power radio-frequency channels for generating ^1H , ^2H , ^{13}C and ^{15}N pulses, and a ^1H - $\{^{13}\text{C}, ^{15}\text{N}\}$ triple resonance cryogenic probe with actively shielded z-gradient. Time domain data size was $125 (t_1) \times 1024 (t_2)$ complex points for both the reference spectrum and the NOE experiment, which were measured in an interleaved manner. Evolution times were $t_{1\text{max}}(^{15}\text{N}) = 74.7 \text{ ms}$, $t_{2\text{max}}(^1\text{H}) = 128 \text{ ms}$, and 20 transients per increment were acquired. 2D [^{15}N , ^1H]-TROSY spectra (32) of the selectively labeled [^{15}N]-Leu]-aKv1.1T1, [^{15}N]-Phe]-aKv1.1T1 and [^{15}N]-Val]-aKv1.1T1 samples, as well as of [70% ^2H , $^{\text{u-}}^{15}\text{N}$]-aKv1.1N samples used for functional studies, were recorded with similar experimental parameters.

The time domain data were processed either with Bruker XWINNMR software or with the program PROSA (33), applying cosine window functions in all dimensions and using zero-filling to twice the recorded data size. Spectral analysis was performed using the programs XEASY (34) and CARA (R. Keller *et al.*, unpublished). Chemical shifts were referenced to 2,2-dimethyl-2 silapentane-5-sulfonate sodium salt (DSS) (35).

Paramagnetic perturbation analysis by NMR

Paramagnetic perturbation analysis and the preparation of the MTSL samples followed methods described by Battiste and Wagner (36,37). Single cysteines were introduced (or reintroduced in the case of amino acid 65) one by one at positions 65, 150 and 166 in aKv1.1N. The resulting variant aKv1.1N proteins were buffered in 20 mM sodium phosphate at pH 7.0, 100 mM NaCl and 1mM [tris(2-carboxyethyl)]phosphine (TCEP). The protein was then diluted into a 10-fold molar excess of MTSL (Toronto Research Chemicals, Inc). Following overnight incubation at room temperature, the excess MTSL was removed by dialysis. The protein was then concentrated by ultrafiltration for the collection of [^{15}N , ^1H]-TROSY spectra. The MTSL was then reduced by the addition of 5mM ascorbic acid to each sample, after which another [^{15}N , ^1H]-TROSY spectrum was collected. Within each pair of spectra, individual peak amplitudes were measured and compared. In each of the three experiments, we observed the entire range of possible effects from the spin label, with peaks that were completely broadened due to proximity, and others that were completely unperturbed. Amplitudes were used instead of peak integrals due to peak crowding. Because of the 4-fold symmetric assembly of the T1 tetramer, discrete distances could not be calculated between the spin label and any given amino acid. Instead, ratios of peak amplitudes were used to derive position information relative to the different probe locations. As an internal calibration control, the perturbation for three amino acids within 10 Å of each probe was calculated.

Electrophysiology

Mutations were introduced into a wild type aKv1.1 construct and verified by sequencing. Constructs were linearized and cRNA transcribed, injected into *Xenopus* oocytes and recorded as described previously (15), except recordings were performed with 98 mM K_o to suppress C-type inactivation, which occurs rapidly following N-type inactivation in low external K_o . Four to six separate recordings were performed for each condition. N-type inactivation and suppression of C-type inactivation were confirmed in these recordings by examining the tail currents during recovery from inactivation. Tails showed a clear N-shape and time course matching the recovery time course as predicted for N-type inactivation. A small amount of C-type inactivation of less than 5% may occur during these recordings, but the much slower recovery from C-type inactivation, time constant $\sim 3 \text{ sec}$, allowed us to separately fit the $>95\%$ N-type recovery. To estimate the energetic effects of these mutations, we calculated an apparent binding affinity, K_A , as the ratio of the inactivation

time constant at +50 mV to the recovery time constant at −100 mV. We then calculated an apparent energy cost for the mutations as $RT \cdot \ln(K_A(\text{Mutant})/K_A(\text{WT}))$.

RESULTS AND DISCUSSION

2D [^{15}N , ^1H]-TROSY correlation spectra of both aKv1.1T1 and aKv1.1N (Figure 2A and 2B) showed single resonances for each amino acid of the monomer sequence, indicating that both proteins are homogeneous, symmetric tetramers. The line widths for aKv1.1N of approximately 42 Hz for ^1H and 27 Hz for ^{15}N are consistent with the large particle size of the tetramer. For the sequential assignment, we used a combination of 3D TROSY-HNCA, 3D constant-time TROSY-HNCA, and 3D ^{15}N -resolved [^1H , ^1H]-NOESY- ^{15}N , ^1H]-TROSY spectra of the uniformly ^2H , ^{13}C , ^{15}N -labeled proteins (Figure 2C and 2D), and 2D [^{15}N , ^1H]-TROSY correlation spectra of the proteins with residue-specific ^{15}N -labeling. For the assignment of the IP and Linker segments of aKv1.1N, which have very limited ^1H chemical shift dispersion, 3D (H)N(COCA)NH spectra (32) provided additional sequential connectivities. The assignment of the polypeptide backbone is nearly complete, except for the residues 18–35. The chemical shift lists have been deposited in the BMRB (accession number 6879).

The C^α chemical shift deviations from the random coil values in aKv1.1N are in good agreement with predictions from the secondary structure of the T1 tetramer crystal structure (Figure 3). The ^{15}N -resolved [^1H , ^1H]-NOESY spectra of aKv1.1N also contain most of the long-range main-chain cross peaks expected from the interatomic distances calculated from the T1 tetramer structure (PDB ID: 1T1D), showing that the T1 domain structure in aKv1.1N coincides with that of the previously determined crystal structure. From a comparison of the [^{15}N , ^1H]-TROSY correlation spectra of aKv1.1N and aKv1.1T1, it is apparent that there is close coincidence of the T1 domain in the two constructs and that the additional resonances of the IP and the Linker in aKv1.1N have narrower line widths and higher intensities than the T1 resonances, indicating shorter correlation times for these residues. The IP and the Linker also have only small deviations from the random coil chemical shifts and show no long-range NOEs, indicating that they do not have a globule-like structure.

These indications of variable mobility along the amino acid sequence of aKv1.1N are confirmed by heteronuclear $^{15}\text{N}\{^1\text{H}\}$ -NOE measurements (Figure 3B). The T1 domain shows positive $^{15}\text{N}\{^1\text{H}\}$ -NOEs, typical of large globular proteins, whereas the IP and the Linker show negative NOE values that indicate intramolecular mobility of these polypeptide segments within the large structure of the aKv1.1N tetramer. Only upper limits of −2.5 could be determined for the chain-termini, and the amino acids 2–13 and 180–181, mainly because of spectral overlap of the corresponding signals in the random coil chemical shift region.

There are a small number of sizeable chemical shift differences for corresponding amino acids between aKv1.1T1 and aKv1.1N (Figure 4A). Other than the N- and C-termini of T1, for which differences are expected due to the changes in the chemical structure, the six amino acids, S73, L81, K82, T83, Y101 and D112, show pronounced changes. A surface rendering of the T1 tetramer (Figure 4B) shows that, although these residues are quite distant from each other in the sequence, they are all located in close proximity on the surface at a cleft between two adjacent monomers of the T1 tetramer. This cleft is large enough (approximately 12 Å deep and 20 Å long) to accommodate a part of the Linker. This surface area of T1 tetramer is thus a prime candidate for being a transient binding site for the IP, the Linker, or both.

To determine whether environmental changes that the cytoplasmic domain may experience during the selective ion flux of the active channel regulate these interactions, we measured

changes of the chemical shifts in response to salt concentration. There are only small chemical shift changes induced in aKv1.1N by increasing the salt concentration from 50 to 400 mM with NaCl or KCl (data not shown). More importantly, there is also no significant cation-specific difference between NaCl and KCl at equal concentrations. These results indicate that aKv1.1N does not undergo any detectable conformational changes or 'disengagement' of the IP or Linker in response to static cation concentration changes. Instead, such changes can possibly be caused by a protein conformational change propagating from the voltage-sensing module of the activated channel or by potassium current induced electrostatic effects.

To better characterize the spatial relationship between the IP, the Linker, and the T1 domain, we covalently attached an NMR broadening agent, the nitroxide spin label 1-Oxyl-2,2,5,5-tetramethyl-Methanethiosulfonate (MTSL), to cysteines engineered into aKv1.1N separately at the amino acids 65, 150, and 166 (indicated in figures 4B and 4C). Positions 65 and 166 are immediately adjacent to the N- and C-termini of the T1 domain. Position 150 is approximately half way between residues 65 and 166, facing outward. MTSL enhances the relaxation of the surrounding resonances, resulting in a loss of peak amplitude, in a distance-dependant manner (36,37). [^{15}N , ^1H]-TROSY spectra of these proteins were compared with control spectra to measure the reduction in peak amplitude (Figure 5). Figure 5 shows ratios of amplitudes for corresponding peaks in the presence and absence of the spin label (see Materials and Methods). Placing the probe at position 150 causes the most substantial loss in the [^{15}N , ^1H]-TROSY spectral signals for residues on the IP. Placing the probe at position 65 results in more signal loss for the IP than when placed at position 166. Amino acids in the Linker become progressively more perturbed by the 65 probe as they approach the junction with T1, as would be expected (data not shown).

As a reference for our analysis of the MTSL labeling, the perturbation of three amino acids within 10 Å of each probe was calculated. Labeling at position 65 broadened peaks from amino acids 62, 63 and 79 on average to the extent of 91%. Labeling at position 150 broadened peaks from amino acids 89, 90 and 152 on average 97%. Labeling at position 166 broadened peaks from amino acids 139, 163 and 164 on average 97%. The spin label experiments thus suggest that the Linker is primarily located near the bottom of T1 (near amino acid 66), whereas the IP appears to be distributed over a wider range of conformational space with respect to T1 (modeled in Figure 4C).

The structural model predicts that binding of the IP and Linker to the T1 domain will reduce the entropic costs associated with IP entry and stability in the pore-binding site. In order to test this model directly, we introduced mutations into the binding site residues K82, D112, and Y101 (Figure 4A). These residues are in close proximity in the three-dimensional structure (Figure 4B). The mutants were characterized for their effects on channel inactivation properties. In order to isolate N-type inactivation from C-type inactivation, recording was performed at elevated external K^+ concentration. Figure 6A compares the inactivation kinetics of wild type (WT) channels to those with the K82A mutation in response to a 1 sec voltage step to +50 mV. Compared to WT channels, K82A slows the kinetics of inactivation and results in a larger level of sustained, non-inactivated current. Upon repolarization, the K82A mutant produces a long transient tail current due to the closing of the non-inactivated channels and the rapid recovery of IP-blocked channels that transits through the open state before closing. The WT current also shows a tail current characteristic of recovery from N-type inactivation, but this current is smaller and slower than for K82A.

In addition to slowing inactivation kinetics, recovery from inactivation is greatly accelerated by the K82A mutation (Figure 6B). Currents were inactivated during a 1 sec pulse to +50

mV, and allowed to recover for varying times at -100 mV before retesting with a second pulse to $+50$ mV. The more rapid recovery of the second pulse amplitude for K82A is clearly apparent. Figure 6C plots recovery kinetic versus time at -100 mV for WT, and for the K82A, D112A, and Y101A mutants. All mutations to this binding site accelerate recovery from N-type inactivation, with K82A having the largest effect.

If we compare the relative effects of these mutations on the time constant to inactivate versus the time constant to recover from inactivation, we can see that there is a clear linear relationship between these parameters: greater slowing of inactivation produces greater acceleration of recovery with a correlation coefficient of 0.96. These results provide clear evidence for a significant functional role of this site in the regulation of channel inactivation and recovery kinetics. The combination of slower inactivation and more rapid recovery of K82A are in line with the higher levels of sustained current seen in Figure 6A. The different quantitative effects of the individual mutations on inactivation are likely due to different energetic effects on the binding affinity of the IP and chain to this site. By taking the ratio of time constants of inactivation to recovery as an approximate measure of binding affinity, we estimate the following energetic costs: K82A: ~ 5 KJ/mol, D112A: ~ 1.4 KJ/mol, Y101A: ~ 0.7 KJ/mol. The central location of K82 in the preinactivation-binding pocket, where it is flanked by amino acids 81 and 83 (Figure 4B), which also exhibited chemical shift changes in the NMR experiments, provides a rationale for the observation that the amino acid replacement in this position has an outstandingly large effect.

In the native channel, the T1 domain constrains access of the N-terminal IP to the pore-lining cavity of the channel. Overall, the present NMR characterization of the isolated tetrameric assembly of the N-terminal 181-amino acid fragment of the *Aplysia* Shaker potassium channel shows that the T1 domain forms a central rigid scaffold, similar to that of the previous crystal structure, around which the disordered and flexible IP and its Linker operate. The narrowness of this pathway and the length of the IP and Linker implicate that the entropic loss of the IP being stretched in an extended conformation into the channel cavity must be compensated by the overall free energy change produced by the binding of the IP. A tightly folded IP or Linker would slow inactivation due to the high energetic cost of unfolding. Therefore, little energy is required for the IP to reach its site of action.

The non-uniform flexibility across the IP and its Linker (Figure 3B) would be consistent with the hypothesis that the presence of a putative preinactivation binding pocket on the surface of T1 may help to reduce the entropic costs of constraining the flexible IP in the tortuous pathway to the IP-binding site within the TM pore domain (3). Furthermore, within the ensemble of IP/Linkers structures only a small fraction need to be bound to T1 at any one time, since only a single IP is sufficient to block the tetrameric channel (11). The experimental data collected in solution suggests that the IP/Linker occupies the pocket on the T1 domain surface, suggesting that this interaction might serve in orienting the IP and thus potentially accelerate the process of inactivation. If the IP is appropriately positioned by interaction with T1, and poised to be drawn into the pore of the channel by the flux of positively charged potassium ions, this will spatially facilitate for the IP to overcome slow random diffusion (38,39). Our results of the largest broadening effects of the IP by MTSL attached centrally about the periphery of the T1 domain are thus consistent with the view that the putative pre-inactivation binding pocket will form the energetic conduit to release the Linker to undergo a conformational change. IP/Linkers bound in this location can account for extra density observed in the low-resolution electron microscopy structure of the full length channel (14). This preinactivation binding of the IP and Linker is in dynamic equilibrium with the fully extended conformation required for inactivation for which voltage-gated channel activation can trigger conversion.

In this study, we provide direct experimental evidence to make two conclusions that help explain the mechanism of inactivation. On one hand, we directly determine that the IP and Linker are structurally flexible. On the other hand, non-uniform flexibility across the IP and Linker segments, and a likely site for interaction on the T1 domain exterior suggest that such transient interaction, may help reduce the entropic costs of IP action. Further support for this model from functional mutagenesis on the T1 domain show that certain mutations to putative surface-exposed residues modulate inactivation kinetics, possibly by disrupting a pre-inactivation site that docks the Linker near the pore opening to inactivate the channel (3, 18). The N-terminal tip of the IP has been previously shown to interact deep within the water-filled cavity region of the channel pore during inactivation (3), therefore, the energetics of conformational change and binding of the IP will play two opposite roles in this inactivation process. In our hypothesis, the IP remains preferentially positioned in its preinactivated state and undergoes conformational change only after the channel becomes activated.

In order to understand more about how the T1 domain is functionally coupled to the remainder of the channel, we will need further information on the flexibility of the linkages between the T1 and TM domains, and on whether or not specific interaction occurs between these two regions of the channel during action. With nearly complete peak assignments available for the aKv1.1N, molecular interaction surfaces of aKv1.1N with other parts of the channel or other cytoplasmic proteins can now be traced if they can be formed as part of a larger covalent complex. The combination of specific structural and functional data on these interactions will be instrumental to characterize further the kinetic mechanisms underlying the regulation of channel activation and inactivation by cytoplasmic domains.

Acknowledgments

Authors thank members of the Choe and Wüthrich laboratories for critical comments and discussion. K.B. acknowledges a fellowship support from the American Heart Association. This work was supported by the NIH (S.C., K.W.).

ABBREVIATION

T1	Tetramerization domain 1
NMR	Nuclear magnetic resonance
IP	inactivation peptide
Kv	voltage-gated potassium (K)
TM	transmembrane
TROSY	Transverse relaxation-optimized spectroscopy
aKv1.1N	N-terminal domain of Aplysia Kv1.1 channel
aKv1.1T1	T1 domain of Aplysia Kv1.1 channel
NOESY	Nuclear Overhauser spectroscopy

References

1. Gonzalez C, Rosenman E, Bezanilla F, Alvarez O, Latorre R. Periodic perturbations in Shaker K⁺ channel gating kinetics by deletions in the S3–S4 linker. *Proc Natl Acad Sci USA*. 2001; 98:9617–9623. [PubMed: 11493701]
2. Hille, B. *Ion Channels of excitable membranes*. 3. Sinauer Associates; Sunderland: 2001.

3. Zhou M, Morais-Cabral J, Mann S, MacKinnon R. Potassium channel receptor site for the inactivation gate and quarternary amine inhibitors. *Nature*. 2001; 411:657–661. [PubMed: 11395760]
4. Yellen G. The voltage-gated potassium channels and their relatives. *Nature*. 2002; 419:35–42. [PubMed: 12214225]
5. Gu C, Jan YN, Jan LY. A conserved domain in axonal targeting of Kv1 (Shaker) voltage-gated potassium channels. *Science*. 2003; 301:646–649. [PubMed: 12893943]
6. Miller C. A charged view of voltage-gated ion channels. *Nat Struct Biol*. 2003; 10:422–424. [PubMed: 12768203]
7. Roosild TP, Le KT, Choe S. Cytoplasmic gatekeepers of potassium channel flux: a structural perspective. *Trends in Biochem Sci*. 2004; 29:39–45. [PubMed: 14729331]
8. Hoshi T, Zagotta WN, Aldrich RW. Biophysical and molecular mechanisms of Shaker potassium channel Inactivation. *Science*. 1990; 250:533–538. [PubMed: 2122519]
9. Zagotta WN, Hoshi T, Aldrich RW. Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. *Science*. 1990; 250:568–571. [PubMed: 2122520]
10. Bezanilla F, Armstrong CM. Negative conductance caused by entry of sodium and cesium ions into the potassium channels of squid axons. *J Gen Physiol*. 1972; 60:588–608. [PubMed: 4644327]
11. MacKinnon R, Aldrich R, Lee AW. Functional stoichiometry of Shaker potassium channel inactivation. *Science*. 1993; 262:757–759. [PubMed: 7694359]
12. Kreusch A, Pfaffinger P, Stevens CF, Choe S. Crystal structure of the tetramerization domain of the Shaker potassium channel. *Nature*. 1998; 392:945–948. [PubMed: 9582078]
13. Bixby K, Nanao M, Shen V, Kreusch A, Bellamy H, Pfaffinger P, Choe S. Zn²⁺-mediated and molecular determinants of tetramerization in Kv channels. *Nat Struct Biol*. 1999; 6:38–43. [PubMed: 9886290]
14. Sokolova O, Kolmakova-Partensky L, Grigorieff N. Three-dimensional structure of a voltage-gated potassium channel at 2.5 nm resolution. *Structure*. 2001; 9:215–220. [PubMed: 11286888]
15. Cushman SJ, Nanao MH, Kunjilwar K, Jahng AW, DeRubeis D, Choe S, Pfaffinger PJ. Voltage-dependent activation of potassium channels is coupled to T1 domain structure. *Nat Struct Biol*. 2000; 7:403–407. [PubMed: 10802739]
16. Choe S, Kreusch A, Pfaffinger PJ. Towards the three-dimensional structure of voltage-gated potassium channels. *Trends Biochem Sci*. 1999; 24:345–349. [PubMed: 10470033]
17. Kobertz WR, Williams C, Miller C. Hanging gondola structure of the T1 domain in a voltage-gated K(+) channel. *Biochemistry*. 2000; 39:10347–10352. [PubMed: 10956024]
18. Gulbis JM, Zhou M, Mann S, MacKinnon R. Structure of the cytoplasmic beta subunit-T1 assembly of voltage-dependent K⁺ channels. *Science*. 2000; 289:123–127. [PubMed: 10884227]
19. Long SB, Campbell EB, MacKinnon R. Voltage sensor of Kv1.2: structural basis of electromechanical coupling. *Science*. 2005; 309:897–903. [PubMed: 16002581]
20. Antz C, Geyer M, Fakler B, Schott MK, Guy HR, Frank R, Ruppersberg JP, Kalbitzer HR. NMR structure of inactivation gates from mammalian voltage-dependent potassium channels. *Nature*. 1997; 385:272–274. [PubMed: 9000078]
21. Schott MK, Antz C, Frank R, Ruppersberg JP, Kalbitzer HR. Structure of the inactivating gate from the Shaker voltage gated K⁺ channel analyzed by NMR spectroscopy. *Eur Biophys J*. 1998; 27:99–104. [PubMed: 9530825]
22. Pervushin K, Riek R, Wider G, Wüthrich K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci USA*. 1997; 94:12366–12371. [PubMed: 9356455]
23. Marley J, Lu M, Bracken C. A method for efficient isotopic labeling of recombinant proteins. *J Biomol NMR*. 2001; 20:71–75. [PubMed: 11430757]
24. LeMaster DM, Richards FM. NMR sequential assignment of *Escherichia coli* thioredoxin utilizing random fractional deuteration. *Biochemistry*. 1988; 27:142–150. [PubMed: 3280013]

25. LeMaster DM, Richards FM. ^1H - ^{15}N heteronuclear NMR studies of Escherichia coli thioredoxin in samples isotopically labeled by residue type. *Biochemistry*. 1985; 24:7263–7268. [PubMed: 3910099]
26. Salzmänn M, Pervushin K, Wider G, Senn H, Wüthrich K. TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins. *Proc Natl Acad Sci USA*. 1998; 95:13585–13590. [PubMed: 9811843]
27. Salzmänn M, Pervushin K, Wider G, Senn H, Wüthrich K. ^{13}C -constant-time ^{15}N , ^1H -TROSY-HNCA for sequential assignments of large proteins. *J Biomol NMR*. 1999; 14:85–88. [PubMed: 10382310]
28. Salzmänn M, Wider G, Pervushin K, Senn H, Wüthrich K. TROSY-type triple-resonance experiments for sequential NMR assignments of large proteins. *J Am Chem Soc*. 1999; 121:844–848.
29. Grzesiek S, Anglister J, Hao R, Bax A. Carbon-13 line narrowing by deuterium decoupling in deuterium/carbon-13/nitrogen-15 enriched proteins. Application to triple resonance 4D connectivity of sequential amides. *J Amer Chem Soc*. 1993; 115:4369–4379.
30. Pervushin K, Braun D, Fernández C, Wüthrich K. ^{15}N , ^1H / ^{13}C , ^1H -TROSY for simultaneous detection of backbone ^{15}N - ^1H , aromatic ^{13}C - ^1H and side-chain ^{15}N - $^1\text{H}_2$ correlations in large proteins. *J Biomol NMR*. 2000; 17:195–202. [PubMed: 10959627]
31. Zhu G, Xia YL, Nicholson LK, Sze KH. Protein dynamics measurements by TROSY-based NMR experiments. *J Magn Reson*. 2000; 143:423–426. [PubMed: 10729271]
32. Pervushin K, Wider G, Wüthrich K. Single transition-to-single transition polarization transfer (ST2-PT) in ^{15}N , ^1H -TROSY. *J Biomol NMR*. 1998; 12:345–348. [PubMed: 21136330]
33. Güntert P, Dötsch V, Wider G, Wüthrich K. Processing of multidimensional NMR data with the new software PROSA. *J Biomol NMR*. 1992; 2:619–629.
34. Bartels C, Xia TH, Billeter M, Güntert P, Wüthrich K. The Program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J Biomol NMR*. 1995; 6:1–10. [PubMed: 22911575]
35. Markley JL, Bax A, Arata Y, Hilbers CW, Kaptein R, Sykes BD, Wright PE, Wüthrich K. Recommendations for the presentation of NMR structures of proteins and nucleic acids. IUPAC-IUBMB-IUPAB Inter-Union Task Group on the Standardization of Data Bases of Protein and Nucleic Acid Structures Determined by NMR Spectroscopy. *J Biomol NMR*. 1998; 12:1–23. [PubMed: 9729785]
36. Battiste JL, Wagner G. Utilization of site-directed spin labeling and high-resolution heteronuclear nuclear magnetic resonance for global fold determination of large proteins with limited nuclear overhauser effect data. *Biochemistry*. 2000; 39:5355–5365. [PubMed: 10820006]
37. Kosen PA. Spin labeling of proteins. *Methods Enzymol*. 1989; 177:86–121. [PubMed: 2558275]
38. Isacoff EY, Jan YN, Jan LY. Putative receptor for the cytoplasmic inactivation gate in the Shaker K^+ channel. *Nature*. 1991; 353:86–90. [PubMed: 1881453]
39. Jiang Y, Lee A, Chen J, Ruta V, Cadene M, Chait BT, MacKinnon R. X-ray structure of a voltage-dependent K^+ channel. *Nature*. 2003; 423:33–41. [PubMed: 12721618]

aKv1.1N Amino Acid Sequence

MEVAMAGIEG	NGGPAGYRDS	YHSSQRPLLA	SSNLPNSASF
PKLSEEDNAN	ENGMGVPGSD	YDSSSERVVI	NVSGLRFETQ
LKTILNQFPDT	LLGNPQKRNR	YYDPLRNEYF	FDRNRPSFDA
ILYFYQSGGR	LRRPVNVPLD	VFSEEIKFY	LGENAFERYR
EDEGFTKEEE	KPLPQNEFQR	R	

FIGURE 1.

Amino acid sequence of aKv1.1N. The IP (amino acids 1-20) and T1 (amino acids 66-165) domains are indicated with open and shaded boxes, respectively. Amino acids differing from the wild-type sequence are R30A, R38A, C63S and C65S, which were exchanged to prevent unwanted proteolysis and slow aggregation. The ovals indicate the three amino acids that were exchanged to cysteine for the purpose of MTSL labeling (see text).

Figure 2A

Figure 2B

Figure 2C

Figure 2D

FIGURE 2.

(A) and (B): [^{15}N , ^1H]-TROSY correlation spectra for aKv1.1T1 and aKv1.1N, respectively. (C) Strips for the amino acids 163-168 from a 3D TROSY-HNCA spectrum of aKv1.1N. (D) Strips for the amino acids 81-86 from a [^1H , ^1H]-NOESY- ^{15}N]-TROSY spectrum of aKv1.1N. Sequential relationships in (C) and (D) are represented by dashed lines. The spectra A through C were measured at 900 MHz, and the spectrum D was measured at 800 MHz, with ~1 mM monomer concentration at pH 7.0 and 37°C.

Figure 3A

Figure 3B

FIGURE 3.

(A) Chemical shift deviations from the random coil values for the C^α resonances of the T1 domain from aKv1.1N. The “Restricted Set of Protein Chemical Shifts, BMRB 2002” were used as the reference random coil chemical shifts. The locations of secondary structure elements identified in the crystal structure of T1 (PDB ID: 1T1D) are indicated by horizontal black bars at +1 for α-helices, and at −1 for β-sheets. (B) Backbone steady-state ¹⁵N{¹H}-NOE values for aKv1.1N. Positive and negative values indicate rigid and dynamic regions, respectively. For the amino acids 2-13 (indicated by an arrow) and 180-181 (not shown), only an upper limit of −2.5 could be determined (see text). The location of the T1 domain is indicated at the bottom by a gray box.

Figure 4A

Figure 4B.

Figure 4C

FIGURE 4.

(A) Normalized chemical shift differences, $(\Delta\delta(^{15}\text{N},^1\text{H}) = [\Delta\delta(^1\text{H})^2 + (0.2 * \Delta\delta(^{15}\text{N}))^2]^{1/2})$ measured between corresponding residues in $[^{15}\text{N},^1\text{H}]$ -TROSY spectra of aKv1.1T1 and aKv1.1N. Shifts greater than 0.07 ppm of non-terminal residues are labeled with the sequence positions 73, 81–83, 101 and 112. (B) The residues with $\Delta\delta(^{15}\text{N},^1\text{H}) > 0.07$ ppm in (A), are mapped in red on the T1 tetramer surface. Adjacent T1 monomers are shaded blue and gold. Positions used for MTSL labeling are marked with magenta circles. (C) Molecular architecture diagram of a eukaryotic potassium channel. The transmembrane and T1 domains from the rat Kv1.2 structure (PDB ID: 2A79) are shown in red, green, blue and yellow. The Linkers of residues 21-65 and the IP are represented as grey and black lines, respectively. The K⁺ conduction pathway is indicated with arrows. The positions used for MTSL labeling are indicated by magenta dots. For clarity, only two diagonally related Linkers and IPs are shown.

FIGURE 5.

Plots of the ratio of the amplitudes of corresponding [^{15}N - ^1H] correlation peaks in a Kv1.1N in the presence of a MTSL spin label (A_{MTSL}), and after ascorbic acid reduction of the MTSL (A_{Quench}) for the N-terminal 17 amino acids of the IP. The broadening arising from the spin label at each of the three positions 65, 150 and 166 (Fig. 4) is represented in light grey, black and dark grey respectively.

Figure 6.

Electrophysiological measurements of N-type inactivation by interaction of IP and Linker with the T1 domain. Alanine mutations to K82, D112, and Y101 were characterized.

Currents were recorded in *Xenopus* oocytes following cRNA injections. **(A)** Currents from K82A channels were compared to wild type in response to a voltage step from -100 mV to +50 mV and back. The K82A mutation delays N-type inactivation and results in a large fraction of non-inactivating current as seen by the large tail current. Scalebar is 200 msec.

(B) Recovery of channels from N-type inactivation is accelerated by K82A mutation as characterized by the more rapid recovery of current amplitude in a second pulse to +50 mV following variable time at -100 mV.

(C) Summary graph showing the accelerated recovery from inactivation for K82A, D112A and Y101A. Y axis is for normalized current.

(D) Summary plot showing that the mutations proportionally slow the rate of inactivation and speed up the recovery from inactivation, suggesting a mechanism with a common energetic basis of action for this site. Linear regression between these points fits with a correlation coefficient of 0.96. Y axis is for recovery Tau in msec.