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Ion Selectivity and Allostery in the NaK Channel

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Ion Selectivity and Allostery in the NaK Channel

By

Joshua Blain Brettmann

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Ion channels require proper ion selectivity and regulated gating in order to perform their cellular functions. Bacterial ion channels serve as excellent model systems to study structure/function relationships concerning the fundamental processes of ion selectivity and gating. NaK, a non-selective cation channel from *Bacillus cereus*, has the conserved pore structure of K+ channels. However, its non-canonical selectivity filter structure leads to non-selectivity between Na+ and K+. Full selectivity is restored with two mutations that lead to the restoration of a Kcsa-like selectivity filter structure. Many mutations can be made to the selectivity filter of NaK without loss of protein stability or function, and this structural stability makes it an excellent model system to study the molecular mechanism of ion selectivity. Experimental measurements on the dynamics and stability of the selectivity filter and how this relates to ion selective have not been performed in this system. The data presented in this thesis establishes that solution NMR dynamic studies of NaK that will lead to a better understanding of how backbone dynamics tune ion selectivity.
The results presented here also reveal an unexpected allostery between the selectivity filter and inner gate of NaK. Allosteric coupling between the selectivity filter and inner gate links gating at the selectivity filter (C-type inactivation) to the state of the inner gate, thus regulating the cycle of channel gating. My work demonstrates that this coupling is an intrinsic structural and dynamic property of NaK and is mechanistically distinct from the well-studied KcsA model. Allostery in channel gating is also important for transmitting signals from regulatory domains to the channel pore. I investigated this coupling of the amphipathic regulatory helix of NaK to the inner gate and found that interaction is mediated through inter- and intra-molecular side chain interactions between M0 and the pore domain. When these interactions are disrupted, the channel reverts to a state similar to what is seen without the regulatory helix.
Chapter I

Introduction and Background

Basic Functional and Structural Features of Ion Channels

Ion channels control the flow of ion across the membrane and combinations of various cellular ion channels allow for complex cellular function, such as shaping of action potentials, and malfunction of any of these activities leads to poor cellular outcomes. Proper ion channel function is dependent on three main functions: selective permeation, ion conduction, and timing of gating. The ion channel pore, which forms the ions conduction pathway, plays a role in all three fundamental functions. For $K^+$ selectivity channels, which are the focus of the work presented here, the conserved pore domain is formed by a symmetric tetramer, with each monomer composed of two transmembrane (TM) helices, a pore helix and the selectivity filter (SF) (Fig 1A). The selectivity filter is held in place by interactions with the pore helix. The two transmembrane helices (termed M1 and M2 or S5 and S6, depending on channel type) are arranged in an inverted teepee, which forms a water-filled vestibule. The selectivity filter and water-filled vestibule form the ion conduction pathway that allows ions to cross the membrane. At the tip of the teepee a hydrophobic gate (the inner gate) is formed by the inner helix (M2 or S6), which prevents ion conduction when in the closed state. Nonselective or $Na^+$ selective pores have similar overall architectures, but with altered selectivity filter sequences and/or an extra pore helix.

Attached to this conserved pore structure are various regulatory domains that modulate channel activity and ensure proper regulation of gating. These regulatory
domains span a wide range of size and complexity, allowing them to be tuned to various stimuli. For the bacterial K⁺ channel model system KcsA, a small pH-sensitive domain is an extension of the M2 helix. Kir channels contain larger cytoplasmic domains that bind small molecules, such as ATP, to trigger channel activity. Voltage-gated K⁺ channels have an extra 4-5 TM helices domain N-terminal to the pore domain which regulate channel activity based on voltage across the membrane. The regulatory domains rarely directly block the ion conduction pathway, instead they act allosterically to open or close the inner gate. My work presented here uses solution nuclear magnetic resonance (NMR) to measure structural and dynamic features in the pore of cation channels and the allosteric communication between regulatory domains and the pore using NaK, a small bacterial ion channel, as the model system.

Bacterial Ion Channels as Models to Study Channel Function

Detailed structural and functional experiments require or are aided by highly purified proteins or molecular systems, and the small size and ease of purification of bacterial channels has made them an attractive target for biophysical and structural studies of channels. KcsA is notable as it was the first crystal structure of an ion channel, thus entering the ion channel field into a new era of structural biology. KcsA was soon followed by KirBac, MthK, the non-selective NaK and the Na⁺ selective channel NavAb as other bacterial ion channels that have been crystalized. Bacterial ion channels are often the first structures solved for their respective classes of channels; thus, these structures have played an important role in understanding structure function relationships for many channels. Sequence alignment and subsequent structures of
eukaryotic ion channels has revealed a conserved structure between eukaryotic and prokaryotic channels, allowing insights gained by studying bacterial channels have been found to be generally transferrable to channels from a diverse range of organisms. Crystal structures only provide a snapshot of a single structural state, however, ion channels are dynamic proteins with multiple structural states that shape the channels’ functions and these dynamics features are poorly understood.

Small bacterial channels have helped bridge the gap from structural snapshot to dynamic function, as purified channels have been studied using a variety of biophysical tools including: patch clamping, single channel bilayer recordings, isothermal titration calorimetry, RB flux assays, single molecule FRET, computational simulations, and NMR. The large quantity of protein able to be expressed and purified from bacterial systems and ease of introducing mutations allow for the wide variety of tools to study bacterial channel function. These biophysical techniques combined with crystal structures have made bacterial channels a system to study: (i) drug binding to K⁺ and Na⁺ channel pores, (ii) effects of toxin binding to K⁺ pores, (iii) mechanism of channel gating, (iv) C-type inactivation in KcsA, (v) lipid specific regulation of KirBac channel, (vi) allosteric communication between gates, (vii) ion selectivity. These findings have helped understand and model functional features of eukaryotic ion channels. Continuing development of these techniques and strategies is important for better understanding of less characterized channels, such as Na⁺, Ca²⁺, and H⁺ channels that do not yet have the same wealth of structural and functional data.
**NaK Channel as a Model System**

NaK is an attractive model system with which to investigate the molecular mechanisms of ion selectivity and channel gating. The wealth of crystal structures of NaK provide a structural framework to develop, test, and interpret results from NMR dynamic experiments. The NaK channel is a non-selective cation channel from *Bacillus cereus* and *Bacillus anthracis* that has high structural homology to K\(^{+}\) selective channels, with the most homology to the cyclic nucleotide-gated (CNG) channels. NaK is permeable to ions in the group 1A elements, as well as some divalent ions such as Ca\(^{2+}\); however, presence of these divalent ions reduce flux of monovalent ions in a similar manner as found in CNG channels. The general structure of NaK follows that of other K\(^{+}\) selective channels with solved structures. For NaK, two transmembrane helices (M1 and M2) create an water filled vestibule in the middle of the membrane that serves as an ion conduction pathway (Figure 1A, 2A). At the bottom of this ion conduction pathway in the closed channel is the inner gate composed of Ile95, Ala99, and Gln103 (Figure 2H). The open channel structure indicates that a twist at a conserved glycine hinge just below the selectivity filter (Gly87) leads to a rotation of the M2, resulting in an opening of the inner gate (Figure 2B, D). Rotation at the hinge leads to rearrangement of a number of hydrophobic and aromatic (Val91, Phe92) residues that serve as intra- and inter-subunit interactions (Figure 2G), leading to opening of the inner gate. This appears to be conserved mechanism, where the NaK closed structure closely matches the KcsA closed structure and its open structure matches the MthK open structure (Figure 2E).
Regulation of NaK is currently unknown; however, NaK has an amphipathic interfacial helix (M0) N-terminal to the M1 helix that runs parallel to the membrane that is a likely candidate for regulation of NaK. The M0 helix is investigated in chapter 5. Interfacial helices are common features for K⁺ channels and often link the pore domain to regulatory elements. However, the M0 helix likely has a direct regulatory role for NaK because no other regulatory domains are attached and it is crystalized in the closed conformation when the helix is present. Removal of this interfacial helix (construct called NaKΔ19 since the first 19 amino acids were removed) causes the protein to crystallize in an open conformation. Functionally, removal of this helix increases the ⁸⁶Rb flux in ion flux assays, suggesting that it plays a role in controlling the state of the inner gate.

The structures of the NaK channel with and without the M0 helix suggest a possible mechanism for coupling the M0 helix to the inner gate; they do not indicate a trigger for this regulation. Two likely interactions between the pore and M0 helix are seen. First, there is a possible complex salt-bridge interaction between Arg10 found on the M0 helix and Glu23 on the M1 helix and Lys97 on the M2 helix of the adjacent monomer (Figure 4A,B). Secondly, on the opposite side of the M1 helix there are further intra-subunit side chain interactions, with a Trp19 capping the M0 helix, forming an aromatic/hydrophobic patch with Phe24 on the M1 helix and Phe94 on the M2 helix (Figure 4A). These inter- and intra-subunit interactions prime the M0 helix to act as a latch to control channel gating.

The structural homology to eukaryotic K⁺ channels, small size, and stability of NaK make it a strong model system to investigate the interplay of structure and dynamics.
in ion selectivity, channel gating, and ion conduction. The remainder of this introduction will detail what is known about: i) the mechanism of ion selectivity, ii) movements involved in ion channel gating, iii) the role of amphipathic helices in conducting signal from the regulatory domain to the inner gate, and iv) allostery connecting the inner gate to the selectivity filter. I will comment on the role of dynamics in these processes and how solution NMR of NaK will help further our understanding of these processes.

**Ion Selectivity**

Potassium channels are able to conduct $\text{K}^+$ ions through the pore across the membrane at a near diffusion-limited rate ($10^7 \text{ s}^{-1}$), yet maintain high selectivity between $\text{Na}^+$ and $\text{K}^+$. This is remarkable as $\text{K}^+$ and $\text{Na}^+$ have similar Pauling radii, 1.33Å and 0.95Å, respectively, and similar charge. This suggests a very unique interaction between protein and ion such that $\text{K}^+$ can be highly selected for without slowing $\text{K}^+$ permeation. While this is a fundamental mechanism for $\text{K}^+$ channel function and has been widely studied, the molecular mechanism of ion selectivity is still unclear.

**Structural perspective of Ion Selectivity**

The KcsA crystal structure revealed a novel structural motif, where the backbone carbonyls in the $\text{K}^+$ selectivity filter signature sequence TVGXG, were aligned to face the center of the pore in a 12Å-long selectivity filter with four ion binding sites (S1, S2, S3, S4) $^2$ (Figure 5). This fourfold symmetric filter is too narrow to fit hydrated ions, necessitating dehydration of ions for permeation. Once the ions are dehydrated they are coordinated by 8 carbonyl oxygen atoms in a square antiprisim geometry with bond
distances from 2.7 to 3.08Å. This structural evidence supports a “snug-fit” model proposed prior to the solving of KcsA. Crystal structures of other K\(^+\) channels support this as a conserved structural motif, identifiable by the signature TVGXG sequence. This model postulates that a rigid selectivity filter can properly coordinate a larger K\(^+\) ion, but the interaction with Na\(^+\) would be insufficient to overcome the energy required to dehydrate the ion unless the structure changed to tightly coordinate the smaller Na\(^+\). Crystal structures of KcsA without K\(^+\) but in high Na\(^+\) do not show densities corresponding to Na\(^+\), rather there is structural collapse of the selectivity filter. This has been suggested as evidence that the selectivity filter of KcsA is unable to deform to accommodate Na\(^+\) while maintaining a conductive state. Four K\(^+\)-binding sites are present inside the selectivity filter, but only two sites are occupied at a time, either S1 and S3 or S2 and S4, as shown by molecular dynamics and crystallography. Further, the fact that K\(^+\) ions are only seen in sites S1/S3 or S2/S4 support a “knock on” mechanism, where repulsion between K\(^+\) ions leads movement of one K\(^+\) ion to push along the K\(^+\) ion at the next site, promoting permeation.

**NaK Contributes to Model of Ion Selectivity**

The non-selectivity between Na\(^+\) and K\(^+\) of NaK compared with KcsA arises from differences in the selectivity filter sequence, TVGDG compared to the TVGYG of K\(^+\)-selective channels (Figure 2). This leads to a selectivity filter with only two ion binding sites, similar to sites S3 and S4 found in K\(^+\) channels, formed by the backbone carbonyl oxygen atoms of Thr63 and Val64 (Figure 5C). In KcsA, hydrogen bonding between a tyrosine and carbonyl groups of the selectivity filter holds the selectivity filter in place.
In NaK, Asp66 extends into the extracellular region of the channel. This hydrogen bonding leads to a reorientation of the backbone carbonyls that make up S1 and S2. Instead of the S1 and S2 sites found in the selectivity filters of K⁺-selective channels, in NaK a vestibule forms above S3/S4 where ions can diffuse but are not coordinated in the traditional carbonyl arrangement (Figure 5 C, D). NaK’s filter maintains the same structure regardless of whether Na⁺ or K⁺ is bound; however, the location of water as well as binding geometry is dependent on the ion identity (Figure 5C, D). K⁺ maintains an octahedral binding in the NaK selectivity filter, with four water molecules coordinating in place of carbonyl oxygen where necessary; this maintains a conductive ion permeation path similar to KcsA. Na⁺ binds in the NaK selectivity filter, but has different coordination. Rather than the octahedral coordination preferred by K⁺, Na⁺ prefers a pyramidal coordination with less-defined binding sites. Unlike KcsA, the selectivity filter of NaK seems stable without the presence of any ions. Studies of NaK’s filter serve as a complement to structural work done with KcsA by showing how water molecules can be used to preserve proper coordination geometry for K⁺, while also being able to also coordinate Na⁺.

NaK is a unique model system to study ion selectivity since the selectivity filter can accommodate numerous mutations, which alter selectivity, without loss of protein stability. This includes a double mutant (D66Y N68D, termed NaK2K) that confers K⁺ selectivity for NaK. These mutations in NaK reorient the backbone carbonyls of the selectivity filter to create four K⁺ ion-binding sites. This reorientation of the NaK selectivity filter leads to a structure that superimposes onto KcsA’s selectivity filter structure within 0.17Å (Figure 5B). However, the individual point mutants leading to
NaK2K are insufficient to form 4 K\textsuperscript{+}-binding sites or confer selectivity. Further mutations in NaK’s selectivity filter suggest that the presence of 4 K\textsuperscript{+}-binding sites is necessary for selectivity, as no structure with less than 4 sites is selective; however, 4 ion-binding sites is not sufficient for selectivity, as one mutant (Y55F) created 4 ion-binding sites and was non-selective (Figure 5E)\textsuperscript{44}. This finding suggests a requirement of four contiguous sites that is not explained by a “snug-fit” model.

Isothermal titration calorimetry has also been used as a tool to study equilibrium binding of K\textsuperscript{+} and Na\textsuperscript{+} ions to both KcsA and NaK\textsuperscript{15,16,55}. Experimenters hypothesized from KcsA’s preference for K\textsuperscript{+} over Na\textsuperscript{+} in equilibrium that selectivity could be explained by a binding preference of K\textsuperscript{+} over Na\textsuperscript{+}\textsuperscript{15,55}. However, similar experiments done with the non-selective NaK also showed an equilibrium binding preference for K\textsuperscript{+} over Na\textsuperscript{+}\textsuperscript{16}. This suggests that equilibrium binding affinities alone cannot explain ion selectivity.

**Importance of the Framework Holding the Selectivity Filter**

The selectivity filter of K\textsuperscript{+} channels is a unique structural motif and requires a scaffold with which to support this filter. A number of experiments illustrate the role of the selectivity filter scaffold in maintaining selectivity: i). In NaK, a mutation (N68D) which is outside of the consensus selectivity filter sequence (TVGXG) is required to form a selective selectivity filter\textsuperscript{46}. Further, a mutation in the pore helix of NaK2K (NaK2K Y55F) also causes a loss of selectivity without changing the structure of the selectivity filter\textsuperscript{44}. ii) There is a wide range of selectivity in K\textsuperscript{+} channels with a conserved selectivity filter sequence; however, these channels have different pore helix sequences\textsuperscript{6}. 
iii) Only some $K^+$ channels with the consensus selectivity filter sequence are able to conduct $Na^+$ ions in the absence of $K^+$. The molecular mechanism that governs the way in which these mutations affect selectivity is not fully understood, but it does suggest that the selectivity filter is able to conduct $Na^+$ at certain times, which is not explained by a snug-fit model.

**Computational Perspective of Selectivity**

The high-resolution structures of ion channels have opened an avenue to studying mechanisms of selectivity via computational methods. An early finding of computational models of ion selectivity was that natural protein backbone fluctuations are greater than the ion size difference between $K^+$ and $Na^+$. This is supported by the B-factors of the 2Å structure of KcsA that indicate greater than 0.75Å fluctuation in the selectivity filter. Free energy perturbations calculations confirm that the filter is selective for $K^+$ over $Na^+$; however, these same simulations have fluctuations in the selectivity filter up to 1Å. This further challenges the validity of the “snug-fit” model, as the selectivity filter can sample conformations in which $Na^+$ could be accommodated.

A number of theories have arisen based on computational models of $K^+$ selectivity. The field strength model developed by Eisenman suggests that ions can be discriminated according to size based on the ligand’s field strength or charge. For $K^+$ channels, this indicates that the low field strength of the carbonyls are optimal for coordination of $K^+$ and less so for $Na^+$. Important to this model is the “liquid-like” coordination by the carbonyl oxygens, where the 8-carbonyl oxygens function like the first coordination shell of the ion in solution. This model works well with a flexible
selectivity filter, and computer binding energies of ion selectivity match well not just for
$K^+$ and Na$^+$ but also other cations that $K^+$ channels can conduct\textsuperscript{57}.

Structural, computational and functional studies of $K^+$ selectivity have not yet
yielded a clear view of the molecular mechanism of selectivity. Crystal structures of $K^+$
channels have supported the “snug-fit” model of selectivity, as well as the importance of
the number of ion binding sites\textsuperscript{48}. The field strength theory of selectivity is supported by
a number computational studies of $K^+$ selectivity; however, a number of other factors
have been proposed to be important in selectivity based on computational studies. These
include dehydration energies for Na$^+$ and $K^+$ to enter the selectivity filter and exact ion
coordination geometry and number\textsuperscript{60}. These methods focus on thermodynamic
equilibrium binding of ions to sites within the selectivity filter, without characterizing the
kinetic factors involved in selectivity\textsuperscript{6}. Importantly, missing from the discussion of
selectivity are measurements of structural and dynamic features of ion selectivity. A
number of factors suggest that selectivity filter dynamics play a role in tuning ion
selectivity: i) fluctuations are seen in the selectivity filter greater than the difference in
diameter between $K^+$ and Na$^+$\textsuperscript{56,58}; ii) the same selectivity filter structure can have
different selectivity\textsuperscript{6}; iii) in certain conditions a selectivity filter that is selective can
coordinate Na$^+$ ions. This thesis will work to use solution NMR to experimentally
measure the role of selectivity filter dynamics and interactions of the selectivity filter
with the scaffold holding it in place.
Ion Channel Gating- Gating at the Bundle Crossing

The location of the main gate, or inner gate, for ion channels is found on the M2 or S6 helix at the point of the inverted teepee where the helices come together and the ion conduction pathway is sterically blocked by a hydrophobic plug \(^1,3\). This gate has been observed with crystal structures of numerous K\(^+\) channels. Computational studies likewise report a high energy barrier for ion conduction through this plug \(^61\). Further, the location of the inner gate was confirmed experimentally several ways: mutations reversing a charged residue at the base of the S6 helix in the Shaker channel lead to a stabilized, open channel \(^62\) and state-based channel accessibility for residues beyond the plug is dependent upon the open state of the channel \(^63\). In NaK the gate has been identified simply from the crystal structure and its the close resemblance to the closed KcsA structure indicates that the inner gate is located at the end of the M2 helix \(^47\). To transition into an open state, a rotation of the M2 or S6 helix about a hinge splays open the inner gate from ~2Å in the closed state to >10Å in the open state (Figure 3). This removes the hydrophobic block and allows for ion conduction. The degree to which the inner gate opens is dependent upon the type of channel, and it is thought that the degree of channel opening may play a role in tuning the single-channel conductance, but this is unclear \(^3\). The splaying is restricted to the lower half of the M2 or S6 helix, and the structural changes in the M1 or S5 helix are minimal, as shown the crystal structures of open and closed channels \(^47\).
Ion Channel Gating- Role of the Hinge

Crystal structures show that the pore helix and selectivity filter are relatively undisturbed by the structural changes at the inner gate. Major structural changes observed crystallographically start at a flexible hinge at a conserved glycine in the M2 or S6 helix, Gly87 for NaK, that is about halfway up the M2 helix near the base of the pore helix (sequence alignment for conservation Figure 2). Mutations of this conserved hinge glycine often disrupt channel function; however, function can be restored by addition of a glycine adjacent to the hinge, which strongly suggests this glycine is functionally crucial to gating movements. Glycine residues introduce kinks and increase dynamics in helices. However, the extent and conservation of this dynamic across various channels has not been measured. Further, flexibility caused by a PXP motif in the S6 helix of many Kv channels, such as the Shaker channels, also contributes to channel opening. The PXP motif leads to additional curvature in the S6 helix in the channel, both in the closed and open state. This extra curvature plays a role in the interaction of the S6 helix with the slide helix that connects the S5 helix to the voltage sensor. This extra disruption of the helix may contribute to channel gating, but the conserved glycine hinge is still important. Measurement of dynamic motions involved in gating, as well as the structural features that regulate the dynamic state of the channels gate, will help elucidate the basics of channel gating, and may help explain the extent to which the hinge rigidity or flexibility affects channel opening. Understanding dynamics at the hinge is important because of its role in gating, single channel conductance, and allosteric communication.
Channel Gating- Allostery from Regulatory Domains

The pore domain contains the ion conduction pathway and is the location of channel gating; however, the signals for gating are often received from attached independent domains, which vary in size and in the signal(s) that they respond to. These regulatory units can be very small, as seen with KcsA, which contains a pH sensing domain, composed of a single helix per monomer, which receives the signal relatively near the gate and hinge. However, regulatory domains are commonly attached via flexible linkers or a slide helix, where the signal is received relatively far from the gate and hinge. This requires allostERIC communication of the gating signal. For some ion channels, large soluble regulatory domains are attached to the N- and/or C-terminus of the pore. These soluble domains are sensitive to a wide variety of signals, from ions, such as Ca$^{2+}$, to small molecules, such as ATP. These soluble domains are connected to the pore via flexible linkers (such as seen with the Rck domains of BK) or by short interfacial helices that lie adjacent to the membrane and help form lipid binding pockets (Kir channels). Further, voltage-sensing domains sit adjacent to the pore domain, connected to the S5 helix by a small amphipathic helix. Channels combine multiple regulatory domains to allow for complex allosteric regulation of the inner gate. For example, BK contains both a voltage-sensing domain and a Ca$^{2+}$-sensing Rck domain, allowing for regulation both by membrane potential and by calcium ion binding.

Remarkably, the wide array of regulatory signals and domains that receive these signals lead to similar conserved motions in the pore of the ion channel. This conservation of motion at the bundle crossing is aided by the fact that the signal is transferred to the pore domain through a similar action of an interfacial helix or linker.
with the S6 or M2 helix. For Kv channels, the S4/S5 amphipathic helix has specific side chain interactions (hydrophobic packing) with the S6 helix below the conserved PXP motif (Figure 6A). Movement of the voltage-sensitive S4 helix is thought to cause the S4/S5 helix to pull on the S6 helix, which opens the inner gate. An amphipathic helix (IF helix) also connects the ATP-binding domain of Kir channels to the pore domain. A complex salt bridge between the M2 helix of the pore and the amphipathic helix mediates signaling from the ATP-binding domain to the inner gate (Figure 6B) and binding of ATP and PIP2 causes the IF helix to pull the inner gate open. In both cases allosteric regulation is mediated by side chain interactions, either hydrophobic packing or salt bridges, between the pore and the regulatory domain. NaK gating is likely regulated by a single helix whose interaction with the pore is mediated by side chain interactions as discussed earlier in this introduction. While the M0 helix interacts primarily through interaction with the M1 helix and not directly with the flexible M2 helix that forms the ion conduction pathway, this system will provide a useful tool to study how changes in the interaction between the interfacial helix and the pore lead to dynamic changes in the hinge and gate.. By measuring dynamics in NaK, it is possible to determine experimentally the structural/dynamic effects of allosteric gating, which cannot be inferred from the available static crystal structures.

**Ion Channel Gating at the Selectivity Filter**

The primary gate for ion channels is found at the constriction point created by the M2 helix; however, C-type inactivation cases loss of ion conduction through structural changes at the selectivity filter. Inactivation serves as a sort of short term memory for
the channel, by preventing the channel from conducting ions on the timescale of seconds. This inactivation was observed as prolonged channel closing following channel opening, and does not occur in all K$^{+}$ channels. Unlike channel gating, inactivation occurs in a stimulus independent way. Structural and functional evidence localizes this inactivation event to the selectivity filter. The mechanism of C-type inactivation has long been a subject of interest; structures of KcsA with a collapsed filter, caused by low K$^{+}$ concentrations, suggest that collapse of the selectivity filter prevents proper K$^{+}$ coordination and conduction. This C-type inactivation is tuned by interactions between the selectivity filter and the pore helix, as shown by a mutation in the pore helix of KcsA (E71A), which leads to a loss of inactivation. In addition, mutations in the pore helix of other K$^{+}$ channels increase the rate of inactivation.

While inactivation happens independently of stimulus, a number of factors likely contribute to inactivation. C-type inactivation is dependent upon loss of K$^{+}$ ions in the selectivity filter in KcsA (dehydration of the selectivity filter has been shown to contribute to filter collapse) and the state of the inner gate, as prolonged periods of opening of the inner gate favor inactivation. Allosteric communication between the selectivity filter and inner gate has been shown experimentally in a number of systems, and has been especially well studied in KcsA with NMR. The allosteric communication between gates is thought to be transferred by interaction between the pore helix and the M2 helix (near the hinge); however, the exact molecular mechanism of this allostery is still unclear. The structural change required for inactivation suggests that the selectivity filter is more dynamic than previously appreciated. Further, inactivated selectivity filters have decreased selectivity between K$^{+}$ and Na$^{+}$, supporting the
hypothesis that the inactivated filter may not be rigid but instead can adopt conformations that are conductive to Na\(^+\). The dynamics of the collapsed filter, however, has not been experimentally measured. C-type inactivation has not been shown for NaK. Data presented in this thesis indicates that allostery exists between the selectivity filter of NaK and the inner-gate and suggests that NaK is a strong model system to measure allostery between the inner-gate and selectivity filter.

**Structure, Function and Dynamics**

Understanding the link between the static pictures given by crystal structures and the dynamic motions required for actions, such as channel opening, allows for a full understanding of the molecular mechanism of channel function. Currently, many structural biology techniques only provide a single snapshot, or snapshots of various states, and thus do not directly provide data on the transitions between states, which is often where important biological activities occur. To fully comprehend protein function in molecular detail it is crucial to study both the populations of conformational states (thermodynamics) and the energy required to transition between states (kinetics). This is done by studying the protein dynamics, which can be described as time dependent fluctuations in protein structure.

Dynamics in proteins occur over a wide range of timescales, with biologically relevant processes occurring in many of these timescales. Large domain motions occur on the ms-s timescales and have large energy barriers between states. This is the timescale of many biological processes, such as enzyme catalysis, protein-protein interactions, and signal transduction (Figure 7). These long-lived states can be
observed directly as a snap-shot from a crystal structure; however, these snapshots are often of kinetically trapped states, and do not provide an idea of the ensemble of states in each energy well. Further, these snapshots provide no information about the transitions between long-lived states. NMR is able to measure both the structures of individual conformations and the kinetics of the transition between conformations at atomic resolution\textsuperscript{78,79}.

Fast fluctuations or dynamics, which fine-tune protein function, occur within the long-lived conformational states and are short-lived, with smaller energy barriers. Local motions and loop motions occur on the nanosecond timescale. Faster motions, such as rotations of side chains, happen on the pico-second timescale (Figure 7). These faster motions are less obviously tied to protein functions, but measurements of dynamics in this time regime highlight hot-spots for protein function. Generally these dynamics are weaker in tightly packed secondary structures; however, secondary structure elements critical to function often show increased dynamics. Experiments with Adk shows that the amino acid sequence tunes the fast time-scale dynamics, which in turn tunes protein function\textsuperscript{80}. As highlighted in the introduction to ion channel function, dynamics are important in a number of channel functions including: i) fast dynamics for ion selectivity (ps-ns), ii) dynamics of ion conduction in the selectivity filter and inner cavity which may tune single channel conductance iii) interconversion of states for channel gating and inactivation (µs-ms), and iv) allosteric communication between regulatory domains and the gate. My work shows that solution NMR using the model system NaK will allow for direct experimental measurements of many of these dynamic features of ion channels.
**Basic measurements of NMR**

Nuclear Magnetic Resonance (NMR) is a powerful tool with which to study both structural and dynamic features of proteins in the same sample, making it a powerful and unique tool for understanding the structure-function relationships of proteins. This section will briefly explain NMR observables that are important for understanding this thesis: i) chemical shift (δ), ii) the intensity (or volume) of the peak, and iii) the linewidth (λ). In order to measure these observables, proteins or other molecules that are enriched in NMR-active nuclei, commonly spin ½ nuclei (1H, 13C and 15N), are used (Figure 8A). Proteins are enriched for these nuclei and purified to high purity and high concentration (generally 0.5-2mM in 0.4mL). These high concentration samples are placed in a high magnetic field (B₀) which aligns the NMR active nuclei along B₀. Radio frequency pulses are then used to tip the bulk magnetization into the transverse plane (x,y), where it will precess in the transverse plane about the B₀ axis as it relaxes back to equilibrium (Figure 8B). The precession rate of each nucleus, or chemical shift, is sensitive to local magnetic fields, and thus reports on local structure, bond torsion angels, ring currents, hydrogen bonding and electrostatics. This precession is measured by a receiver coil, which records the free induction decay (FID), the sum of all the individual nuclei in the sample (Figure 8C). A fourier transform shifts the information from the time domain to the frequency domain, creating the NMR spectrum with peaks corresponding to the precession frequency, or chemical shift, of each nucleus (Figure 8D, E).

Most of the spectra presented in this work are two-dimensional NMR experiments (1H-15N-HSQC) which correlate the chemical shift of bonded nuclei (15N-1H bond) to
give resolved peaks, compared to simple 1D spectra (Figure 8E). The sensitivity of chemical shifts to their environment creates many uses of this information such as: i) Secondary structure calculation using the chemical shift information of the amide NH, Cα, Cβ, and C’, which are sensitive to torsion angle and amino acid type and ii) changes in local structure or binding of ligands by analyzing changes in in the N and H chemical shifts. While the chemical shift is sensitive to structural elements of the protein, it cannot be used alone to solve a structure, as there are too many contributions to the signal to analyze. The second major reporter in NMR is the peak volume of the signal, which reports on the number of nuclei or relative population of nuclei in that particular environment. The final observable for NMR studies is linewidth, which is defined as the peak width at half the maximum peak height. Line-width is sensitive to protein dynamics in the ps-ms regime and can be used to measure dynamics in that time regime. Chemical shift, peak intensity and linewidth are all important NMR observables that allow for the study of both structure and dynamics of proteins and are fundamental to this thesis work.

Assignment of NMR Spectra

Atomistic resolution is a powerful feature of NMR, but requires assignment of each peak in the 1H-15N-HSQC to the particular amide in the protein. This step of the NMR process is challenging and time consuming, especially for large proteins that have many nuclei and fast relaxation. This section will give a brief outline of strategies of assignments that are tuned for large systems.
In order to assign the peaks in the N/H correlation plots, the first step is to link the peaks that are sequential in the primary sequence. This is done by walking along the backbone (Figure 9) with 3-D spectra (ex: HNCA, HN(Co)Ca). An HNCA spectrum measures the H/N chemical shift information in much the same manner as the 2D correlation spectrum already explained, but also measures the correlated \( C_\alpha \) chemical shift information of each amino acid’s own (i) and preceding \( C_\alpha \) atom (i-1) (Figure 9A). The HN(Co)Ca measures the H/N and i-1 \( C_\alpha \), allowing for determination of which \( C_\alpha \) peak corresponds to the i and i-1 peaks in the HNCA. This provides sequential information to “walk” along the backbone; however, peak overlap, proline (which do not have an amide bond), and poor signal can disrupt this walk.

Backbone walks for large proteins are especially fraught with difficulties, as the increased number of peaks increases spectral overlap, and the large size leads to slower tumbling and more relaxation (thus weak or lost peaks). To overcome these limitations, higher field strength magnets can be used to increase signal and resolution. Specially designed methods, including TROSY (Transverse relaxation optimized spectroscopy)\(^{86}\) and BEST (Band-Selective Short Transient)\(^{87,88}\), have been designed to increase signal and thus decrease the time required to collect lengthy backbone walk experiments. Using non-uniform sampling, where only a sampling of points in the indirect dimension is acquired, and used to reconstruct the full spectrum, can further reduce experimental time\(^{89}\). A final method that aids in assigning large proteins involves amino acid-selective labeling\(^90\). The most useful technique here is to label all amino acids with \(^{15}\)N and add in the \(^{14}\)N/\(^{13}\)C\’ amino acid of interest (Figure 10). This effectively removes all specifically-labeled amino acids from the \(^{15}\)N/\(^{1}\)H correlation spectra and reveals only these amino
acids in the $^{15}$N/$^1$H plane of an HNCO, which will show the amide of the amino acid following the selectively $^{13}$C' labeled type (Figure 10 A,C). Specific labeling can be done for a subset of amino acid types, but is limited by metabolic processes that will scramble the labels from one amino acid type to another. However, the limited number of amino acids that can be labeled give good points in the backbone walk with high confidence of the assignment. This thesis includes the initial spectra of NaK, along with the assignment of NaK, NaKΔ19 and other NaK mutations.

**NMR and Protein Dynamics**

Dynamic measurements for NMR on the $\mu$s-s timescale are generally broken up into three main categories: fast time scale dynamics, where exchange between states ($k_{ex}$) happens faster than the difference in frequency (or chemical shift, $\Delta\nu$) between the exchanging states ($k_{ex} >> \Delta\nu$); slow exchange ($k_{ex} << \Delta\nu$) and intermediate exchange ($k_{ex} \approx \Delta\nu$) (Figure 11). In fast exchange there is a single peak representing the population-weighted average of the chemical shifts of all states present. A single peak is seen in this case because exchange occurs during the frequency detection periods, leading to a single averaged signal. In contrast, slow exchange has a distinct peak in the spectrum for each state, as exchange is slower than the frequency detection of the experiment. Peak intensities for each exchanging pair correspond to the population of each state. If exchange occurs at a rate near the difference in frequency ($k_{ex} \approx \Delta\nu$), then the chemical shift will be averaged between the two states, but extreme line-broadening will occur.79,82 Quick initial observations about protein dynamics are available for N/H correlation NMR spectra: i) peak doubling likely indicates the presence of at least two population
with slow dynamic exchange between them (however, it is possible that there are two populations without exchange), and ii) differential peak intensity between peaks in the spectra indicate that dynamics are occurring on the intermediate (causing exchange broadening) and fast time scales. These techniques will allow for simple identification of dynamics, but in-depth studies of protein dynamics rely on more sophisticated methods.

Two such sequences were used in my work to examine the dynamic underpinnings of NaK function. Carr-Purcell Meiboom-Gill (CPMG) experiments measure exchange in the 0.3-10ms time frame and have been widely used to study dynamic exchange in proteins and small molecules. For systems of interest in this thesis, this will involve important channel functions such as movements involved with channel gating at the hinge. In CPMG pulse sequences, 180° pulses are separated by relaxation delay periods (t). If exchange occurs within the delay period, the 180° pulse will not refocus the signal sufficiently, and signal broadening will occur. By keeping a constant time in which 180° pulse trains are applied, but changing the delay period and number of pulses (CPMG frequency, \( v_{\text{CPMG}} \)), an intensity decay curve is generated. The relationship between the \( v_{\text{CPMG}} \) and the observed relaxation at each \( v_{\text{cpmg}} \) can then be fit to give the exchange rate and difference in chemical shift between the states. Determination of exchange for a two-state exchanging system is relatively straightforward; however, for states with more than two exchange processes, determination of the effective difference in relaxation (\( \Delta R_{2,\text{EFF}} \)), gives a good estimate of areas with exchange in this regime, as shown in chapter 3.

Dynamics faster than detected by CPMG also play an important role in many biological functions. Especially of interest in this thesis is how these ps-ns dynamics can
help tune ion selectivity for NaK. Fluctuations in the selectivity filter that would be relevant to coordinating K$^+$ vs Na$^+$ would likely be very small (~1 Å) and occur on a fast timescale. This timescale is also interesting, as ion conduction through the selectivity filter would occur in this regime. Nuclear spin relaxation reports on these ps-ns dynamics. Unlike dynamic experiments previously discussed, dynamics this fast do not cleanly occur between two well-isolated states; rather, these are motions that occur within an energy well. Three measurements are done to measure dynamics in this regime: i) measuring longitudinal relaxation ($R_1$, or relaxation occurring with recovery of magnetization back to bulk magnetization) ii) transverse relaxation ($R_2$, relaxation occurring due to loss of coherence of the spins while in the x,y plane) iii) through-space magnetization transfer (HetNOE) between $^1$H and $^{15}$N. Measurements of these three relaxation parameters can then be fit to give an order parameter to identify regions of the protein with increased dynamics in this time regime. While it is ideal to collect all these relaxation parameters, this is not always possible for large, slow-tumbling systems, and subsets of these relaxation parameters can be interpreted individually. For rigid proteins, there should be little difference in fast dynamics throughout the non-loop regions. However, if dynamics play an important role in protein function, this will likely be shown by an increase in dynamics in the region of the protein involved in the protein function. For ion selectivity, I hypothesize that selectivity filters that are non-selective display increased dynamics, as they are less-stiff and can accommodate ions of different size.
NMR in KcsA

NMR, either solid or solution, has been rarely used to study ion channels, with most work done on KcsA, M2 proton channel, or isolated domains of ion channels (HCNQ1 domain, or soluble domains of ion channels)\textsuperscript{23–31,92–96}. Studies have largely been hindered by poor NMR peak dispersion and intensity in these systems, as well as the difficulty of isolating these systems in membrane mimetic environments. As NaK is largely homologous to KcsA, this introduction will focus mainly on NMR done on KcsA. KcsA was first studied in solution NMR by Ad Bax’s group, and assignment of KcsA in SDS micelles was the first assignment of an ion channel with solution or solid state NMR\textsuperscript{33}. In SDS, KcsA largely maintains the secondary structure expected; however, the harsh detergent environment may have caused folding issues for the pore helix\textsuperscript{27,33}. KcsA was later assigned and studied in n-dodecylphosphocholine, and coupling between the selectivity filter and gate was seen\textsuperscript{32}. Further work was done for KcsA using solid state NMR, which allows for study of membrane protein in liposomes, most closely mimicking the conditions of the membrane\textsuperscript{23,25,28,31}. Here, conformational exchange similar to the rate of channel gating was shown in KcsA by solid state NMR\textsuperscript{97}, similar to what was seen by Imai et al with solution NMR studies\textsuperscript{26}. Further, allosteric coupling between the selectivity filter and inner gate has also been clearly shown with KcsA using solution NMR. A change in protonation state or mutation of the intracellular gate induces a change in equilibrium at the extracellular gate\textsuperscript{24}. In addition, residues in the selectivity filter shifts with pH similar to residues in the inner gate even though there are no residues that would be pH sensitive in the selectivity filter\textsuperscript{32}. Allosteric coupling has also been shown by the appearance of similar chemical shifts for the inner gate for low pH (channel
open) and high pH, low K⁺ (channel inactivated) via solid state NMR. Increased line-broadening for KcsA mutant E71A, which leads to decreased channel inactivation as well as decreased K⁺ selectivity, suggests a role of dynamics in ion selectivity. Since the less-selective mutant has more line-broadening, this indicates more structural dynamics or a structurally heterogeneous filter for the less selective mutant. The generality of these findings is difficult to assess because, to my knowledge, all NMR studies of the pore of K⁺ channels have examined KcsA and KcsA hybrids. Examination of a second system with different selectivity would help to determine if allosteric coupling and selectivity filter dynamics are important for a variety of channels. This work provides an initial assessment of the dynamics of the non-selective NaK and a selective mutant (NaK2K0 solubilized in isotropic bicelles, and allows for the study of NaK in a membrane-like environment.

**Scope of Thesis**

In the Chapters that follow, I will detail the work that I have performed during my graduate studies. I will start by describing the important methods that are generally used in subsequent chapters. Chapter 3 presents the assignments for NaKΔ19, and shows that allosteric communication between the selectivity filter and the inner gate is inherent to the NaK channel, which previously has not been shown. Chapter 4 describes preliminary data on the role dynamics play in tuning the selectivity of NaK and NaK mutants. Chapter 5 reports on allosteric coupling between the M0 helix and the inner gate is then examined, showing that interactions between the M0 helix and the M1 helix alter the
state of the inner gate. Together, these findings demonstrate novel allostery in NaK and show the potential to NaK to study the role of protein dynamics in ion selectivity and allosteric gating.

References


Figure 1. Basic Structural Features of Ion Channels. A. General structure of a K⁺ channel pore domain, shown as dimer with monomers opposite each other shown. Regions discussed in this thesis are mapped by color on the NaK structure (PDB: 2AHZ): M0 helix (green), M1 helix (Orange), Pore Helix (Magenta), Selectivity filter (Red), and M2 helix (Blue). B. Topology diagrams of major families of K⁺ channels. Pore domain colored cyan and pink. Attached regulatory domains are shown in tan or green. Figure from McCoy, J. Nimigean, C. 2012. (6)
**Figure 2.** Sequence Alignment of NaK and Similar K⁺ Channels. This alignment shows the sequence similarity of NaK with commonly studied K⁺ channels in the M1, pore and M2 helices. This alignment also shows the sequence divergence of the selectivity filter. The first arrow shows where the NaK∆19 construct starts. The second arrow indicates the conserved glycine hinge. Figure from Shi, N. et al., 2006. (5)
Figure 3. Structural Rearrangements between Open and Closed Structures of NaK. **A.** Open NaK structure with the glycine hinge highlighted in red and the pore blocking F92 in green. **B.** Overlay of open (blue) and closed (red) NaK structures. The selectivity filter region is unchanged. **C.** Intracellular view of NaK pore in open (blue) and closed (red) structures. **D.** Same view as **C,** with helices as cylinders. Rotation of helices and change in angle are labeled. **E.** Similarity of the open (blue) and closed (red) state of NaK with KcsA (yellow, PDB 1K4C) and MthK (orange, PDB 1LNQ) viewed from the intracellular side. **F.** Detailed interactions in the closed structure of NaK (red). **G.** Detailed interactions in the open structure of NaK (blue). Figure from Alam & Jiang. 2009. (47)
Figure 4. Interactions between the M0 Helix and Pore: from a side view (A) and from the intracellular side (B). Aromatic and hydrophobic patch is highlighted in red and the complex salt bridge in green. (PDB: 2AHZ).
Figure 5. Structure of the Selectivity Filter. A. Ion binding to the KcsA selectivity filter. The four ion binding sites are labeled 1-4. This same labeling follows on subsequent filter structures in this figure. K$^+$ ions are coordinated by the backbone carbonyl oxygen in a similar manner to how water coordinates them outside the filter. B. NaK2K has the same structure as KcsA, and is K$^+$ selective. C, D. Structure of the selectivity filter of NaK in K$^+$ (C) and Na$^+$, the use of water coordinating ions differs between Na$^+$ and K$^+$. (D). E. Selectivity filter structure of NaK2K Y55F. This filter adopts the same structure as NaK2K, but is non selective between Na$^+$ and K$^+$. Figure A-D from Alam & Jiang. 2011 (40) Figure E from Sauer, D. et al., 2011. (44)
Figure 6. Interactions between the Pore and Interfacial Helices in Kv2.1 (A, PDB 2A79) and KirBac (B, PDB 3SPI). Interfacial helices are in red and M2 or S6 residues that interact are in green.
Figure 7. Energy Diagram and NMR Dynamics. A. Simplified energy diagram showing different energy wells where exchange occurs. Large wells with large energy barriers are slower exchanging. Within these large wells are faster exchanging states with low energy barriers. Crystal structures capture only one stable state and does not measure the faster dynamics. B. Important motions occurring at different time regimes and the corresponding NMR experiments to measure the exchange. Figure from Kleckner and Foster, 2011. (79)
Figure 8. Fundamental Measurements with NMR. A. $^{15}\text{N}/^{13}\text{C}/^{1}\text{H}$ labeling of the protein backbone. The amide bond is circled as this bond leads to the correlation spectra in E. B. Illustration of measuring chemical shift. NMR-active nuclei are knocked into the XY plane by an external magnetic field. The precession of the nuclei in this plane is recorded by a receiver coil. The magnetization is recorded as a free induction decay (FID) show in C. which contains all the chemical shift frequencies of all the NMR-active nuclei in the sample. D. A fourier transform in performed on the FID to give spectra in the frequency domain. This frequency domain spectra can be very crowded, so multi-dimensional NMR helps alleviate crowding (E). B,C,D are adapted from lecture of Katie Henzler-Wildman.
Figure 9. Three Dimensional NMR and Protein Backbone Walk. A. $^{15}$N/$^{13}$C labeled protein use 3D pulse sequences to correlate chemical shifts of i and i-1 residues in the protein backbone (as shown by red arrows on protein backbone). B. These experiments measure chemical shifts in N/C/H dimension creating a 3D box of shift information. C. Pull $^{15}$N strips and “walk” along the $^{13}$C chemical shifts connecting i to i-1. This connection is shown as green dashed lines. Red peaks correspond to $^{13}$Cβ, and black peaks to $^{13}$Ca.
Figure 10. Amino Acid Specific Labeling to find anchor points for assignment. B. Labeling scheme for $^{1-13}\text{C}^{14}\text{N}$ amino acid labeling for glycine where all other amino acids are $^{15}\text{N}$ labeled. In this scheme, the C’ of the labeled amino acid is $^{13}\text{C}$ labeled, with other C and N unlabeled. The unlabeled N causes these peaks to drop out of $^{15}\text{N}/^{1}\text{H}$ HSQC (A). i+1 residues are identified by the $^{12}\text{N}/^{1}\text{H}$ plane of an HNCO, where magnetization is started on the amide H and transferred to the i-1 C’. Only residues i+1 of a labeled C’ have a peak as shown in C.
Figure 11. Exchange Regimes in NMR. Slow, intermediate and fast exchange is determined by the relationship between the exchange rate ($k_{ex}$) and chemical shift difference between the states ($\Delta \nu$). Characteristic features allow for some initial determination of dynamics in a spectrum. Figure from Kleckner and Foster, 2011. (79)
Chapter II

Materials and Methods

Expression and Purification of NaK

Two different lengths of NaK were used in this Thesis, the full-length NaK containing the M0 helix, and NaKΔ19 constructs that are missing the first 19 residues (Chapter 1 Figure 2)\(^1,2\). For Chapters 5-6, the appropriate NaK or NaKΔ19 will be used to describe the construct. For Chapter 3-4 all experiments were collected with NaKΔ19 but to highlight the mutants used, NaK is used to describe NaKΔ19. The constructs and mutants sequences were checked with genewiz.

NaK and the mutants studied here were purified largely as published\(^3,4\) and the protein prep was the same for NaK and NaKΔ19, with a few changes to optimize expression in minimal media. NaK was transferred from NaK-pQE60 (generously provided by Youxing Jiang) to a pET15B vector with an N-terminal 6xHis tag. Spectra were taken of both NaK and NaKΔ19 in the pQE60 vector. These spectra indicate that the overall fold is maintained, but the spectral quality was poor. NaK in the pET15B vector had 2-3 x higher yield of NaK, when expressed in minimal media for isotopic labeling. BL21(DE3) cells containing the NaK-pET15b were grown in LB media with ampicillin. After two hours the culture was transferred to 1 L of M9 media. For isotopic labeling, the standard media components were substituted with \(^{15}\text{NH}_4\)Cl, \(^{13}\text{C}\)-glucose, or D\(_2\)O plus 0.5 g/L isotopically labeled isogro (Sigma-Aldrich). Cells were induced with 0.4 mM IPTG at OD 0.8-0.9 and harvested after overnight expression at 25 °C. Cells were stored at -80°C if purification was not performed immediately. To purify NaK, cells...
were resuspended in lysis buffer (100 mM NaCl, 200 mM KCl, 2.5 mM MgSO₄, 20 mM tris pH 7.5, DNase, 1µg/ml pepstatin, 10 µM leupeptin, 100 µM PMSF) with 250 mM sucrose, 1mg/ml lysozyme, and lysed by sonication. The membrane fraction was isolated by spinning for 1hr at 30,000 g, resuspended in lysis buffer with 20 mM decylmatoside (Anatrace), and solubilized for 3 hrs with rotation at room temperature. Insoluble protein was removed by centrifugation at 30,000 g for 10 min. Solubilized NaK was purified by IMAC with 0.5 mL Talon cobalt affinity resin (Clontech) per liter of growth that had been pre-washed with Buffer A (5 mM DM, 20 mM Tris-HCl, 200 mM KCl, 90 mM NaCl, pH 7.8). NaK was allowed to bind for 20 minutes at room temperature and then the beads were washed with 10 bed volumes of Buffer A followed by 10 bed volumes of Buffer A with 15mM imidazole. NaK was eluted in 8 bed volumes of Buffer A containing 300mM imidazole, concentrated to 0.5 mL and loaded on a Superdex 200 column equilibrated in NMR buffer (100 mM MOPS 40 mM KCl pH 7) containing 5 mM DM. For low K⁺ and low salt samples, gel filtration chromatography and all subsequent steps of the protocol were performed using 100 mM MOPS/40 mM NaCl for low potassium or 100 mM ultrapure MOPS for low salt. To cleave the 6xHis-tag, fractions containing NaK were incubated overnight with 10 U of thrombin (Sigma).

**Reconstitution of NaK into Isotropic Bicelles.**

NaK concentration was determined by A280 using the calculated extinction coefficient of 3840 L•mol⁻¹•cm⁻¹ for NaKΔ19 and 9590 L•mol⁻¹•cm⁻¹ for full length NaK. NaK was reconstituted into bicelles using our previously published protocol with slight alterations ³. Briefly, DMPC (1,2-dimyristoyl-sn-glycerol-3-phosphocholine) was
hydrated at 20 mg/ml in NMR buffer, bath sonicated ~1 min and solubilized with 10 mM DM for 20 min. Cleaved NaK was added to the solubilized DMPC at a molar ratio of 1:100 NaK monomer/DMPC and rotated at room temperature for 3hrs. Two aliquots of 45 mg Amberlite XAD-2 (BioRad) per milligram of total detergent were added to remove the detergent and incubated overnight at room temperature. After the Amberlite was removed, the NaK proteoliposomes were centrifuged at 40,000 rpm for 2 hrs at 6°C. The proteoliposome pellet was solubilized with DHPC dissolved in NMR buffer to create a final ratio of 1:3 DMPC:DHPC. Four freeze thaw cycles produced uniform q=0.33 DMPC/DHPC bicelles containing NaK, which were flash-frozen and stored at -80°C until use.

**Rubidium Flux Assay**

Liposome reconstitution procedures and flux assay were carried out as described before with some modifications. In short, purified NaK was reconstituted into a 10 mg/ml lipid mixture containing 3:1 1-palmitoyl-2-oleoyl-phosphatidylethanolamine:1-palmitoyl-2-oleoyl-phosphatidylglycerol dissolved in a reconstitution buffer B (450 mM KCl, 10mM HEPES, 4mM NMDG, 1mM EDTA and 1mM EGTA, pH 7.4) containing 32.5 mM CHAPS for lipid solubilization. Protein was mixed with lipids in ratio of 10 ug mg⁻¹ and incubated for 20 mins at room temperature. Proteoliposomes containing NaK were formed by centrifuging the protein/lipid mixture through a 1.5-mL Sephadex G-50 gel filtration columns to remove the detergent. For flux to occur a electro-chemical gradient was formed by exchange the outside buffer to buffer C (400 mM Sorbitol,10mM HEPES, 4mM NMDG, 1mM EDTA and 1mM EGTA, pH 7.4) which had low salt. The
uptake mixture was made by addition of liposome samples to $^{86}$Rb flux buffer containing 5uM of hot rubidium in buffer C taken in calculation 400ul per sample. To remove extra-liposomal $^{86}$Rb 60-80 ul aliquot of the uptake mixture was passed over a 1.5–ml Dowex cation exchange column at each time point. After loading on Dowex columns samples were eluted twice with 1 ml of 400mM Sorbitol into scintillation vials. For normalization, valinomycin at a final concentration of 1 ug ml$^{-1}$ was added to the remaining uptake mixture samples not passed through Dowex columns, incubated for 10 min, and then the corresponding aliquot was loaded on and eluted from Dowex column as described above. All eluted samples were mixed with 16 ml scintillation fluid, and radioactivity was measured in a scintillation counter. This assay was used to determine if NaK was functional and is discussed in Chapter 3.

**NaK Assignment**

A major part of this Thesis work was to assign the backbone of NaKΔ19 and NaK. Assignment of these spectra, and a number of the NaK and NaKΔ19 mutants opens the door to many future projects with these proteins. Both NaKΔ19 and NaKFL were assigned in a similar matter, using a combined strategy of traditional backbone walk experiments (Chapter 1 Figure 9C), amino-acid-specific labeling to provide anchor points (Chapter 1 Figure 10) $^{6}$, and amide NOESY. The technical challenges of assigning such a large (8 transmembrane helix) membrane protein solubilized in isotropic bicelles were overcome by taking advantage of the thermal stability of NaK and recent developments in non-uniform-sampling (NUS) and BEST-TROSY NMR $^{7,8}$. For BEST-TROSY backbone walk experiments, 10% of data points were collected with a poison gap.
generated schedule. The NUS data was reconstructed using the iterative soft threshold (istHMS) reconstruction method.

For NaKΔ19, without deuteration, 87 out of 95 expected non-proline resonances are observed. As was observed for KcsA, full deuteration of NaK leads to a loss of ~40% of peaks due to incomplete back-exchange within the core of the transmembrane helices, consistent with a stably folded structure. Using used D2O that was distilled, and fully deuterated isogro, an estimated ~70% deuteration gave a good mix of number of peaks as well as sufficient deuteration for strong signal in the backbone walk. A similar deuteration scheme is used for NaK and NaKΔ19. Assignment of 83% of the non-proline residues was achieved for NaKΔ19, and ~70% for NaK, as the increased overlap in NaKFL lead to less assignments. Assigned residues for NaK and NaKΔ19 are shown Chapter 3.

**NMR Experiments**

NMR samples contained 0.5-1.5 mM NaK in q=0.33 DMPC/DHPC bicolles in 100 mM MOPS 40 mM KCl pH 7 (or other buffers as specified in the text) with 10% D2O. ¹H-¹⁵N TROSY spectra were collected on a 700 MHz Varian spectrometer with a room temperature probe. Three-dimensional backbone walk experiments were acquired on a 600 MHz Bruker spectrometer with cryoprobe (Washington University) or 750 MHz Bruker spectrometer with cryoprobe (NMRFAM). TROSY-HNCA, BEST-TROSY HNCA, BEST-TROSY HN(co)CA and BEST-TROSY HNCACB were collected using a non-uniform10% poisson-gap sampling schedule, reconstructed using IST-HMS, processed using nmrpipe and analyzed with CCPNMR. The backbone walk
experiments were supplemented with 1-13C'-amino acid specific labeling. Assignments were submitted to the BMRB. Chemical shift differences ($\Delta\delta_{tot}$) were calculated using equation 1,

$$\Delta\delta_{tot} = \sqrt{(\Delta\delta_H)^2 + (0.154\Delta\delta_N)^2}$$

TROSY-CPMG experiments were performed on an 800 MHz Varian spectrometer with a cryoprobe, with a relaxation delay ($T_{relax}$) of 40 ms. Two CPMG field strengths were acquired with a low frequency $n_{CPMG}=100$ Hz and high frequency CPMG of $n_{CPMG}=1000$ Hz. $\Delta R_{2,app}$ was determined with equation 2, with $I_{n_{CPMG}}$ and $I_0$ the peak intensities with and without CPMG refocusing periods. $\Delta R_2$ was calculated for all well-resolved residues using equations 2 and 3.

$$R_{2,app} = -\frac{1}{T_{relax}} \times \ln \left[ \frac{I_{(v_{CPMG})}}{I_0} \right]$$

$$\Delta R_2 = R_{2,app}(v_{CPMG}=100\text{Hz}) - R_{2,app}(v_{CPMG}=1000\text{Hz})$$

HetNOE experiments were performed on a 800 MHz Varian spectrometer with a cryoprobe. They were collected as two interleaved 3D spectra (NOE and reference) with a 8 second delay to establish steady state prior to each scan. NOE value was calculated using the ratio of the signal intensities from the NOE and reference experiment using CCPNMR.
References


Chapter III

Role of Protein Dynamics in Ion Selectivity and Allosteric Coupling in the NaK Channel

This Chapter was submitted to PNAS August 2015. Upon submission of this thesis, no decision has been made on its publication. I did the major portion of the work in this chapter, with Darya Urusova collecting the $^{86}$Rb flux data, and Tonelli, Marc collecting the CPMG.

Abstract

Allosteric communication is crucial for proper gating in potassium channels. The relationship between channel opening and C-type inactivation has been well characterized in KcsA. Here we present NMR data demonstrating structural and dynamic coupling between the selectivity filter and intracellular constriction point in the bacterial non-selective cation channel, NaK. This transmembrane allosteric communication must be structurally different from KcsA, since the NaK selectivity filter does not collapse under low cation conditions. Comparison of NMR spectra of the non-selective NaK and potassium-selective NaK2K indicates that the number of ion binding sites in the selectivity filter shifts the equilibrium distribution of structural states throughout the channel. This finding was unexpected given the nearly identical crystal structure of NaK and NaK2K outside the immediate vicinity of the selectivity filter. Our results highlight the tight structural and dynamic coupling between the selectivity filter and the channel scaffold, which has significant implications for channel function. NaK offers a distinct model to study the physiologically essential connection between ion conduction and
channel gating with many advantages over the KcsA model.

**Significance**

Pore domains of ion channels form the ion conduction pathway and thus control the identity and flux of ions across the membrane. Flux-dependent inactivation suggests that these two functions are connected, and KcsA provides one mechanistic model for allosteric linkage between the selectivity filter and inner gate of a channel. This study uses solution NMR to investigate structural and dynamic features of ion selectivity and allosteric communication in the small bacterial channel NaK. NaK is a non-selective cation channel that is homologous to several eukaryotic channels that are not well modeled by KcsA. Our results show a previously unexpected allosteric linkage between the selectivity filter and inner gate in NaK. This suggests that such transmembrane allosteric regulation is widespread.

**Introduction**

Small bacterial ion channels are often used to study the detailed molecular mechanisms underlying fundamental channel functions, such as gating and selectivity. Being the first membrane protein crystal structure \(^1\), KcsA became the primary model system for investigating the relationship between channel structure and ion conduction and has proven to be a robust structural model for many eukaryotic channels \(^2\)–\(^6\). In particular, transmembrane allostery between the cytoplasmic channel gate and the selectivity filter has been established as the basis for channel inactivation in this model system \(^6\)–\(^12\). While allostery between gates is functionally conserved in many channels, not all channels have the same properties as KcsA. More recently, the NaK channel from
*Bacillus cereus* has been developed as a second model system with distinct functional properties \(^{13–17}\). Although the overall structure of the pore region is similar in NaK and KcsA (Fig. 1A), there are distinct differences in the selectivity filter sequence \((_{63}TVGDGN_{68})\), leading to only two ion binding sites in the non-selective NaK channel (Fig. 1B) \(^{1,17}\). Only two mutations are required to convert the NaK selectivity filter sequence to the KcsA sequence, creating the NaK2K construct \((_{63}TVGYGD_{68})\) \(^{16,17}\). This leads to K\(^+\)-selectivity and a selectivity filter structure nearly identical to KcsA with four ion-binding sites. Outside of the immediate vicinity of the mutations in the selectivity filter, the high-resolution crystal structures of NaK and NaK2K are essentially identical with an all atom RMSD of only 0.24Å.

In addition to its role in determining ion selectivity, the selectivity filter plays an important part in channel inactivation. Flux-dependent inactivation is physiologically important in many eukaryotic channels \(^{18–22}\) and recent studies of KcsA have provided a structural basis for inactivation through collapse of the selectivity filter. This model also explains the strong correlation between channel selectivity and inactivation \(^{21}\).

Crystallographic, functional, and MD simulations suggested allosteric coupling between the inner gate and selectivity filter, with inner gate opening leading to selectivity filter collapse and inactivation \(^{6,21,23}\). More recent NMR studies tested this model, directly comparing demonstrating that the low pH and low K\(^+\) states of KcsA are very similar \(^{7,9–12,24,25}\) even though these triggers act at the inner gate and selectivity filter, respectively. As might be expected with such transmembrane allosteric coupling, the scaffold surrounding the selectivity filter is critical for stabilizing particular selectivity filter conformations and determining the selectivity and inactivation properties of KcsA \(^{21,26}\).
Further, interaction between the side chains at the base of the selectivity filter and pore helix at the conserved M2 hinge support the role of the scaffold and filter as a part of the allosteric connection\textsuperscript{26}.

NaK offers a distinct model to study the physiologically essential connection between ion conduction and channel gating. The NaK selectivity filter structure is identical in Na\textsuperscript{+} or K\textsuperscript{+}\textsuperscript{15}, consistent with its non-selective behavior, and there is no evidence of selectivity filter collapse in the many high-resolution NaK crystal structures now available, including no collapse of the selective NaK2K filter in low ion conditions\textsuperscript{27}. Here we use NMR spectroscopy to test whether transmembrane allosteric coupling between the inner gate and selectivity filter can occur in NaK. Surprisingly, we find significant differences in the NMR spectra of NaK and NaK2K that extend throughout the protein and are not localized to the selectivity filter region. This suggests that the allosteric communication between the selectivity filter and the inner gate may occur in NaK or is reintroduced upon mutation to the canonical selectivity filter sequence.

**Results**

**NaK is functionally reconstituted in isotropic bicelles and is suitable for solution NMR.** NaK is small and highly stable, making it a good model for solution NMR studies. We confirmed that NaK produced in our lab was functional using radioactive rubidium flux assays\textsuperscript{28} (Fig. S1). NaK reconstituted into liposomes was then solubilized into lipid bicelles by our previously published protocol for solution NMR experiments\textsuperscript{29}.

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Protein NMR studies usually begin with acquisition of 2D-\(^1\)H, \(^{15}\)N chemical shift correlation spectra. These spectra provide insight into both the structure of the protein through the observed chemical shifts and peak dispersion, as well as protein dynamics through the number of peaks and their relative intensities. The TROSY-HSQC spectrum of \(^{15}\)N-NaK solubilized in 1:3 DMPC:DHPC bicelles at 45°C displays excellent peak dispersion (Fig. 2), indicating that NaK is well-folded under these conditions. Helical membrane proteins often have significant peak overlap due to the modest dispersion of amide chemical shifts in helical regions and the uniformity of the membrane environment. The good resolution observed for NaK suggests that the helices are tightly packed in a well-defined tertiary structure leading to unique chemical shifts. Following assignment of 83% of NaK peaks at 65°C, the secondary structure of NaK determined by the chemical shift index matches the crystal structure (PDB 3E8H) quite well (Fig. 2 inset)\(^{30,31}\). This confirms the structural stability of NaK in isotropic bicelles up to 65°C, as expected from its thermal stability, and establishes that NaK in isotropic bicelles is suitable for detailed investigation of channel structure and dynamics via solution NMR.

**The NaK selectivity filter does not collapse.** Spectra of NaK in bicelles with K\(^+\) only, Na\(^+\) only, and low salt conditions show very minor shifts and these shifts are localized to the selectivity filter and adjacent regions (Fig. 3). This confirms that the selectivity filter of NaK is stable, maintaining the same structure regardless of bound ion, and does not collapse in low ion conditions. This is consistent with previous crystallographic data\(^{15,27}\). NMR chemical shifts are exquisitely sensitive to the surrounding environment, including both through-bond and through-space effects. Small chemical shifts in the selectivity filter (D66 and G67) and surrounding loops (S70)
reflect the effect of having a different ion bound to the channel since these residues are all in close proximity to the ion conduction pathway and ion binding sites. This establishes that NaK solubilized in isotropic bicelles binds ions, as required for a properly-folded functional channel.

**Selective NaK has perturbed chemical shifts for residues far from mutations.** To study the structural and dynamic effects of the selective NaK2K, we collected $^{15}$N-TROSY-HSQC spectra. In the high-resolution crystal structures of NaK and NaK2K, differences are highly localized to the selectivity filter region (Fig. 1C)\(^{17}\). We therefore expected relatively few peak shifts when comparing TROSY-HSQC spectra of NaK and NaK2K bound to $K^+$, and that only peaks in and around the selectivity filter would have unique chemical shifts in the two constructs (Fig. 1B,C). Instead, we observed significant chemical shift changes for many peaks (Fig. 4A) corresponding to residues throughout the protein. This indicates that significant structural or dynamic differences exist between NaK and NaK2K that were not predicted from the x-ray structures. Due to the significant chemical shifts differences between NaK and NaK2K, additional 3D backbone walk NMR spectra were acquired to assign NaK2K.

Mapping $^{15}$N and $^1$H chemical shift changes from NaK to NaK2K onto the NaK structure reveal that the largest chemical shift changes are indeed located in the selectivity filter region as expected (Fig. 4C). A second set of significant shifts are seen at the hinge of NaK below the selectivity filter, including residues G87 and G89 (Fig. 4A,C). Further down the M2 helix, smaller but still significant chemical shift changes occur at F94 and I95. These residues are part of a hydrophobic patch that rearranges upon inner gate opening in the crystal structures\(^{14}\). A final set of chemical shift changes are
see near N101 and Q103, the channel constriction point, which also undergoes structural rearrangement upon channel opening. This constriction point is over 25 Å away from the site of mutations, well beyond the range of simple effects of mutation on neighboring residues or structural differences predicted by the crystal structures. Our data shows that the sequence and structural state of the selectivity filter affects the entire NaK protein. These chemical shift changes far from the point of mutation may be explained by a shift in protein structure that is not captured in the crystal structure, or they could arise from changes in equilibrium protein dynamics.

Such allosteric coupling between the selectivity filter and the inner gate is well established in KcsA and several other K⁺ channels, but has not previously been observed in NaK, which has a much more stable structural scaffold. This stability is demonstrated by the minimal chemical shift changes observed in low salt (Fig. 3), which is in marked contrast to the large chemical shift differences between NaK and NaK2K. Individual mutations leading to NaK2K (NaKD66Y and NaKN68D) have less significant chemical shift changes, and the effects are much more concentrated in the vicinity of the selectivity filter (Fig. 4B). Neither of the single mutants is selective¹⁷, suggesting that formation of a four site K⁺-selective filter may be important for allosteric coupling.

**Structural and dynamic changes between NaK and NaK2K.** Backbone carbon chemical shifts and the secondary structure determined from the chemical shift index using TALOS+ are very similar between NaK and NaK2K (Fig. 5C)³¹. However, significant amide chemical shift differences between NaK and NaK2K exist in the hinge point and constriction point. While the crystal structures represent a high-resolution snapshot of a single state, the single set of peaks observed in the NMR spectrum reflect
the population weighted average of all the states sampled by NaK. This assumes relatively fast sampling between different structural states, a reasonable assumption given the flicker nature of channel gating observed for NaK and NaK2K \(^{17}\) and the relatively high temperature of the NMR experiments. This would suggest a shift in the equilibrium distribution of structural states between NaK and NaK2K, leading to the observed chemical shift differences. An alternative interpretation of the NMR spectra is that NaK has only a single major structural state and this state changes between NaK and NaK2K. This excludes protein dynamics and seems less likely given the similarity of the crystal structures.

Initial assessment of dynamic differences between NaK and NaK2K was performed by comparing peak intensities between the two spectra. Increased protein motion leads to line-broadening and a decrease in peak intensity. For comparison, peak intensities were normalized to the C-terminal residue, N110, which is far from the site of mutation and has identical chemical shifts in NaK and NaK2K (Fig. 4A). NaK2K has stronger peak intensities in the region around the selectivity filter, particularly for residues in the pore helix (I51, L54), but also in the surrounding loops (Fig 5A, B). Outside the scaffold surrounding the selectivity filter differences in peak intensity between NaK and NaK2K are less significant, with an increase in peak intensity for NaK at the constriction point. This suggests that the selectivity filter region and surrounding scaffold of the K\(^{+}\)-selective NaK2K channel is less dynamic or more structurally homogenous than that of the non-selective NaK. This data is consistent with a role for structural stability in achieving ion selectivity, a phenomena that has been extensively studied previously \(^{32}\).
**Transmembrane allostery is inherent to NaK.** The data presented thus far indicate that the sequence and structure of the selectivity filter are communicated through the scaffold behind the selectivity filter to the hinge point, and across the membrane to the intracellular gate. However, this comparative analysis of NaK and NaK2K cannot determine whether such transmembrane allostery is present in NaK or whether it is simply re-introduced when the 4-site selectivity filter is inserted. The significant differences in line width and peak intensity suggest a role for dynamics on the µs-ms timescale. We therefore tested whether NaK itself had dynamics on this timescale using 15N-TROSY-CPMG experiments (Fig. 6A,B). Due to the inherent insensitivity of these experiments, full deuteration of the protein is required and this reduces the number of residues that can be monitored. The figures show $\Delta R_2$, determined as described in the methods. If there is no exchange present on the µs-ms timescale, this should be 0. If there is conformational exchange on this timescale, $\Delta R_2$ will be positive. Since there is no reason to assume 2-state exchange a priori and the available electrophysiology data on NaK are not sufficient for quantitative comparison of rates and populations we did not attempt to further analyze the exchange process quantitatively in the current work. The experiments presented here do demonstrate that there is conformational exchange throughout the protein, particularly at the bottom of the selectivity filter and the hinge point in M2, as well as at the constriction point on the intracellular side of the channel. Since CPMG experiments detect dynamics only when the exchanging states have different chemical shifts, not every residue in a region that is moving will have a measurable $\Delta R_2$. The regions where the most significant $\Delta R_2$ values are observed are exactly where local structural changes occur between the open and closed channel (Fig. 6A,B).
5A,B). These results support the idea that transmembrane allostery between the selectivity filter and intracellular gate is an inherent property in NaK.

**Discussion**

**Implications for Ion Selectivity.** The structural stability of NaK has enabled engineering of the selectivity filter, tuning the number of ion binding sites and ion selectivity of the channel. Beautiful high-resolution crystal structures of these different mutants have contributed to our current understanding of the structure-function relationships underlying ion selectivity.\(^{15-17,27,33}\) Here we present NMR characterization of the NaK channel, demonstrating that NaK is also well-suited for NMR studies that add experimental data on dynamics.

The scaffold holding the selectivity filter has long been appreciated as playing an important role in tuning selectivity in K\(^+\) channels.\(^{6,21}\) NMR studies of KcsA show line broadening in the selectivity filter and scaffold for a mutation that decreases selectivity.\(^{25}\) Our results similarly demonstrate that the scaffold behind the selectivity filter in NaK is more rigid when the selectivity filter has the canonical K\(^+\)-selective sequence and structure and less rigid with the nonselective NaK sequence and structure. Previous studies of NaK led to the model that four ion-binding sites are necessary for potassium selectivity through a knock-on mechanism.\(^{34,35}\) Together with our data, this suggests that structural stability is important for achieving selectivity because a more rigid scaffold is needed to stabilize the 4-ion binding site filter structure required for selective ion conduction.
**Transmembrane allostery in NaK.** Significant chemical shift changes between NaK and NaK2K are seen throughout the protein, revealing allostERIC coupling between the selectivity filter and inner gate. In KcsA, where such transmembrane allostery is well-established, it has been suggested that the base of the selectivity filter and pore helix are critical for this behavior. Despite differences in the sequence and detailed atomic structure of NaK and KcsA in this region, our data support similar coupling between the selectivity filter and inner gate through the M2 hinge point in NaK. However, there is no indication of selectivity filter collapse (Fig. 3), a key feature associated with allosteric coupling in KcsA. Thus, transmembrane allosteric coupling must be structurally different in NaK than it is in KcsA. Flicker gating in NaK suggests a role for the selectivity filter in channel gating, however the electrophysiological function of NaK is not as well studied. Thorough single channel recordings of this system combined with more quantitative NMR studies will be needed to resolve the role of the selectivity filter in flicker gating and the mechanism linking the selectivity filter to the inner gate.

CPMG NMR experiments reveal that μs-ms timescale motions occur at the hinge and constriction point as well as the base of the selectivity filter in NaK. This provides a dynamic pathway connecting the selectivity filter to the inner channel gate. Motion on this timescale is often correlated with exchange between functional states in the case of enzymes. The data presented here cannot assign the exchange process to channel opening/closing, but it is interesting to note that the residues sensing motion on this timescale are localized to the regions of NaK that would change environment upon channel opening/closing.
Interestingly, the regions with μs-ms dynamics in NaK are the same regions with the largest chemical shift changes between NaK and NaK2K. This suggests that formation of a rigid K⁺ selectivity filter leads to changes in the equilibrium state of the M2 hinge and the inner gate. There are limited chemical shift changes for the two individual point mutants (D66Y, N68D) that lead to NaK2K, consistent with the idea that major shifts in equilibrium dynamics connecting the selectivity filter with channel gating correlate with selectivity. Our results emphasize the intimate connections between the selectivity filter and the overall channel structure. These interactions are important for both structural stability of the selectivity filter and dynamic coupling between the filter and inner channel gate, which will determine the functional properties of the ion channel.

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**References**

Figure 1. Crystal structures of the non-selective cation channel NaK and the potassium-selective NaK2K mutant show structural changes restricted to the area of the selectivity filter. **A** The selectivity filter region of the NaK crystal structure (PDB 3E8H) has only two ion binding sites fully coordinated by backbone carbonyl groups, corresponding to sites 3 and 4 of the canonical KcsA selectivity filter structure. **B** Alignment of the WT NaK (blue) and NaK2K (red, PDB 3OUF) selectivity filters shows a KcsA-like four ion-binding-site selectivity filter is created by the NaK2K mutations (D66Y, N68D). **C** Alignment of the full NaK (blue) and NaK2K (red) structures shows no structural changes outside the vicinity of the selectivity filter. Two (A,B) or three monomers (C) from the tetramer are shown for clarity.
Figure 2. $^{15}$N-TROSY HSQC NMR spectra of NaK in isotropic bicelles reflect a stable, well-folded protein. A) Spectra of NaK in 3:1 DHPC:DMPC isotropic bicelles at 45°C (black) and 65°C (magenta) show only minor chemical shifts changes, reflecting the high thermal stability of NaK. The inset structure shows the secondary structure predicted from the backbone chemical shift index using TALOS+ (31) mapped onto the NaK crystal structure (PDB 3E8H) with alpha helical regions colored blue, loops red and unassigned residues grey. Three monomers of the channel are shown for clarity.
Figure 3. Ion induced chemical shift changes are specific to the selectivity filter region of NaK. $^{15}\text{N}$-TROSY HSQC spectra of NaK in the presence of 40 mM KCl (black), 40 mM NaCl (red) and very low salt ($\approx 0.2$ µM KCl, green). Only small chemical shift changes are observed, demonstrating the structure of NaK does not change significantly in the presence or absence of bound ions. Residues that do sense the identity of a bound ion or its absence are localized to the selectivity filter, $^{63}\text{TVGDG}^{68}\text{N}$, including D66, G67, F69, and S70.
Figure 4. NMR spectra reveal differences between NaK and NaK2K are not localized to the selectivity filter but extend throughout the channel. A) Comparing $^{15}$N TROSY-HSQC spectra of NaK (black) and NaK2K (red) reveals widespread amide chemical shift changes. These differences occur for many more residues than would be expected based on the crystal structures (Fig. 1). Insets highlight residues in the M2 helix far from the selectivity filter mutations that create NaK2K. B) NMR spectra of each of the individual point mutants (D66Y in green, N68D in orange) have much less dramatic chemical shift changes than NaK2K, and these changes are much more localized to nearby residues in the vicinity of the selectivity filter. C) The chemical shift differences between NaK and NaK2K are plotted on the NaK structure (PDB 3E8H) on a blue to red color scale. This highlights the significant chemical shift changes extend throughout the M2 helix, including the hinge region and hydrophobic patch near the intracellular constriction point, regions where structural changes occur between the open and closed channel.
Figure 5. Changes in peak intensity between NaK and NaK2K reflect dynamic differences in the scaffold behind the selectivity filter. A) Plot of the difference in peak intensities between NaK and NaK2K. Positive values indicate more intense peaks in NaK. Peaks that are unassigned, overlapped or in the mutated region were excluded from analysis and the secondary structure is indicated at the top (selectivity filter in red). B) Residues with more intense peaks in NaK2K are mapped onto the crystal structure (PDB 3E8H) in blue, residues with more intense peaks in NaK are shown in red. Residues with no significant change are colored yellow and residues that are unassigned, overlapped, or very close to the points of mutation are colored in gray. More intense peaks in NaK2K are consistent with a more rigid scaffold supporting the selectivity filter in this mutant. C) $\text{C}_\alpha$ Chemical shift differences between NaK and NaK2K are plotted on the NaK structure on a blue to red color scale. The few large chemical shifts, which are only in the regions adjacent to the mutations, confirms that there are no major secondary structure rearrangements.
Figure 6. Dynamics on the μs-ms timescale in NaK extend across the membrane. A) $\Delta R_2$ ($s^{-1}$) measured with a two-point TROSY-CPMG NMR experiment. Residues were excluded if they were too weak for quantitative analysis, unassigned or overlapped. The secondary structure is indicated at the top (selectivity filter in red). B) $\Delta R_2$ ($s^{-1}$) data plotted onto the NaK structure on a blue to red color scale. Dynamics on the μs-ms timescale are detected in the selectivity filter as well as the M2 hinge region and intracellular constriction point, regions where conformational change occurs upon channel opening/closing.
Figure S1. NaK is functionally active when purified from Pet15B vector. \(^{86}\text{Rb}\) flux assay of NaK in 3:1 POPE:POPC liposomes. Two time point show an increase in rubidium flux indicating a functional channel.
Figure S2- High quality 3D spectra allow for backbone walk of NaK. Strips of the backbone walk of NaK showing Best-TROSY: HNCA (black), HN(Co)Ca (green) and HNCaCb (red). Backbone walk shown for $^{100}\text{VNVQL}_{104}$. This backbone walk was complimented with amino acid specific labeling to provide anchor points.
Figure S3- $^{15}$N-$^1$H assignments of NaK. A. Completed assignments of NaK plotted labeled on NaK spectra. B. Assignments for crowded region of NaK spectra. Assignments were completed as described in results and method.
Figure S6. Secondary structure of NaK2K matches crystal structure. Calculated secondary structure predicted from the backbone chemical shift index using TALOS+ mapped onto the NaK2K crystal structure (PDB 3OUF) with alpha helical regions colored blue, loops red and unassigned residues grey. Three monomers of the channel are shown for clarity.
Chapter IV

Backbone Dynamics and Ion Binding to the NaK Ion Selectivity

Josh Brettmann, Katie Henzler-Wildman

This chapter contains unpublished work that I have done. I performed and designed the majority of the work presented here. Heteronuclear NOE experiments were collected at the University of Wisconsin, Madison with the help of Marco Tonelli.

Introduction

Ion selectivity is a critical channel function that requires high fidelity ion interaction and fast conduction of ions across the membrane ($10^7$ s$^{-1}$). The molecular mechanism through which the selectivity filters fulfills both these requirements is not well understood. Chapter 1 introduced the current models of ion selectivity, this chapter shows how solution NMR studies, using NaK as a model system, can investigate two main features of ion selectivity: i) site specific ion-selectivity filter interactions and ii) the role of backbone dynamics in ion selectivity.

Computational simulations of ion conduction through the selectivity filter shows that conduction of Na$^+$ across the S2 site in the filter is much less favorable than K$^+$ conduction across this site, however the molecular mechanism for this is unexplored$^{1,2}$. ITC suggests that this selectivity is not due to equilibrium binding affinity$^3$. In this chapter I show that the site-specific resolution of solution NMR can investigate interactions between the selectivity filter and ions by monitoring chemical shift changes in the selectivity filter upon ion binding. The backbone of selective filter is critical in coordinating the ions. Thus, the backbone carbonyl carbon and the adjacent amid bond
are likely to be sensitive to ion binding and are candidates to measure ion-binding\textsuperscript{4-6}. The structural stability and wealth of high-resolution crystal structures of NaK allow for study of ion binding in various selectivity filter sequences/structures, and will aid in interpreting any site specific binding preference\textsuperscript{6-13}.

The rigidity of the selectivity filter likely contributes to the ability of the selectivity filter to properly select between Na\textsuperscript{+} and K\textsuperscript{+}. As outlined in the Chapter 1, the “snug-fit” model of selectivity suggests a highly rigid filter, yet this is not supported by computational simulations of the backbone motions in the filter.\textsuperscript{4,5,14,15} Further, differential degrees of selectivity for channels with identical filter sequences also suggests that dynamics are important for tuning ion selectivity\textsuperscript{16}. This is seen for NaK, as a mutant of NaK2K (Y55F) preserves the conserved four-site K\textsuperscript{+} selective filter structure, but is not selective for Na\textsuperscript{+} over K\textsuperscript{+} (Chapter 1 Figure 5)\textsuperscript{13}. This loss of selectivity for this mutant is attributed to an increase in dynamics due to the loss of a hydrogen bond behind the selectivity filter, but this has not been experimentally verified. Work in this chapter shows that fast backbone dynamics can be measured for NaK and will discuss how these measurements will significantly contribute to our understanding of ion selectivity. Further, it examines NaK2K Y55F and shows this mutant is useful in testing the hypothesis of the role of dynamics in selectivity.

Results

Ion Binding to the Selectivity Filter of NaK

To measure specific Na\textsuperscript{+} or K\textsuperscript{+}-selectivity filter interactions in NaK, I reconstituted NaK in 40 mM KCl, 40 mM NaCl, and low ion conditions (<0.2\textmu M either NaCl, KCl). This data was presented in Chapter 3 Figure 3, to show that there is no major
structural collapse in NaK like as is seen in KcsA \(^{17}\). These spectra indicate that small changes are indeed seen in a number of residues in the selectivity filter (Asp66, Gly67, Ser70), suggesting these residues are sensitive to ion concentration and identity. Small shifts are seen for Asp66 and Gly67. Interestingly, Ser70 show the largest chemical shift change even though it is in the extracellular loop just above the selectivity filter (Figure 1 A, C) and does not show bound ions in the high-resolution crystal structures. Fitting a titration of increasing K\(^+\) shows a Kd of 10 uM (Figure 1 B), which is similar to what has been estimated as the binding constant via ITC \(^3\). However, the chemical shift changes are not linear for increasing K\(^+\) concentration, which means this is not a simple binding reaction and additional data is needed to properly fit this data.

Unfortunately, Asp66 and Gly67 did not show a titration curve in the K\(^+\) concentration range used and no chemical shift changes were seen for other residues in the selectivity filter. This may be because the amide chemical shift was measured. The amides do not directly coordinate the ions, and without a structural change to the filter, the local environment of the amides in the backbone my not change enough to measure. The chemical shift of the backbone carbonyl carbon, measured by \(^1\text{H}-^{13}\text{C}\) planes from an HNCO experiment, would be more likely to show significant chemical shift changes. However, poor signal to noise prevented analysis of the carbonyl carbon chemical shift information. Higher field strength, deuteration and optimized pulse sequences may help recover the lost signal.

**NaK2K is Unstable without K\(^+\)**

A similar titration was attempted with the selective NaK2K, however, NaK2K is unstable in isotropic bicelles with low K\(^+\). Na\(^+\) was unable to replace K\(^+\) and stabilized
NaK2K, and no other ions were tested for stabilizing NaK2K selectivity filter. Because NaK2K is stable in detergent without K⁺, in both my hands and in published work via x-ray structures and ITC, its stability is dependent upon a lipid-like environment. It also seems unlikely that NaK2K is inherently less stable than NaK because they have similar stability (stable at 65°C for weeks) in bicelles in K⁺ containing buffer. This suggests the selectivity filter of NaK is intrinsically more stable than that of the selective NaK2K, and that a deformed selectivity filter for NaK2K, which might form without the presence of K⁺, has effects for the whole protein. Reconstitution in low K⁺, instead of no K⁺, may allow for study of selectivity filter interactions in NaK2K and should be pursued.

**NaK2K Y55F Similar to NaK2K Below the Gate**

The selectivity filter structure of a K⁺ selective NaK channel is very conserved, with a full four-ion coordinating sites needed to confer selectivity. Interestingly a single NaK mutant (NaK2K Y55F) crystalized with the conserved four-ion binding sites, but is non-selective. The mechanism through which this four-site selectivity filter is non-selective, while all other selectivity filters with the conserved structure are selective is not understood. Jiang’s group suggested that this mutant was non-selective in part due to loss of the conserved H-bond between Tyr55 and Asp68 (Chapter 1 Figure 5), which may lead to a more dynamic filter and correspondingly to a loss of selectivity. A study of the structural and dynamic changes in this mutant, which has high backbone structural similarity to NaK2K, but is non-selective, will help verify the role of dynamics in ion selectivity.

For initial assessment of this mutant a ¹H/¹⁵N TROSY-HSQC was collected. There were no obvious dynamic difference observed in this mutant, however, TROSY-
HSQC’s are only sensitive to dynamics in the us-s regime. With assignments known for both NaK and NaK2K, many assignments could be transferred to NaK Y55F, however, 3D backbone walk spectra of this mutant will be needed to assign ambiguous resonances. Mapping chemical shift changes of NaK2K Y55F between NaK and NaK2K suggests that the selectivity filter region of NaK2K Y55F is more similar to NaK than NaK2K (Figure 2B,C). This is seen by the similar chemical shifts of R49, L82, I51, and is clearly seen in the chemical shift map in figure (Figure 2A-C). While the M2 helix of NaK and NaK2K Y55F are similar, the M1 helix in that region has more chemical shift changes. The nearness of the mutation to the selectivity filter region complicates this analysis in this region. The chemical shift changes in NaK2K Y55F further supports allostery between the selectivity filter and inner gate, which was discussed in chapter 3.

**Picosecond-nanosecond Dynamics in NaK2K**

Measurement of the fast dynamics occurring in the backbone of the selectivity filter will aid in the understanding the molecular mechanism of ion selectivity. To measure the dynamics in the ps-ns time range we collected $^1$H-$^{15}$N heteronuclear NOEs (hetNOEs) for NaK2K and these are plotted in Figure 3. For well-structured regions of the protein the hetNOE values will be high, near 1, and disordered regions will be low. High hetNOEs in the M1 helix suggest this helix is structurally very stable, and low hetNOEs in loops suggest increased disorder (ex: E46, G47) as expected. Sparse assignments in the pore helix combined with the poor signal to noise leaves little signal in the pore helix, however, hetNOEs are high in the selectivity filter, suggesting a stiff filter. Lower hetNOE values were measured in the M2 helix compared to the M1 helix, especially at the hinge (G87) suggesting the M2 helix involved in gating is less rigid than...
the M1 helix, which maintains a similar structure in the two states. The increased
dynamics measured in the M2 hinge suggest that it may be primed for dynamic exchange,
similar to dynamics seen in the M2 helix in Chapter 3. HetNOEs are a single
measurement of fast dynamics. Further measurement of T1 and T2 relaxation, combined
with the hetNOEs measured, will allow for calculation of the order parameter in the
selectivity filter and additional analysis. Similar fast dynamic measurements for the non-
selective NaK were attempted, but high quality spectra were not obtained due to technical
difficulties.

**Discussion**

**Ion-Protein Interactions in the Selectivity Filter**

Due to the requirement of high fidelity ion interactions and fast conduction,
examination of specific ion-protein interactions will help determine the molecular
mechanism of ion selectivity. Solution NMR provides a technique to do this, by
measuring chemical shift changes as ion concentration or ion identity change. Solution
and solid state NMR has shown chemical shift changes with ion concentration in KcsA,
however, this channel undergoes selectivity filter collapse, and thus will have a larger
chemical shift change than NaK. For NaK, the chemical shift changes are localized
near the selectivity filter as expected, and are much smaller than what is seen for KcsA.
The limited number of small shifts is consistent with a stable filter in the absence of ions
(discussed in Chapter 3). The nonlinear titration of S70 shows that multiple processes
are happening at S70 as ions bind. Fitting of this titration reveals a binding affinity
consistent with the micromolar affinities found via ITC. The lack of shifts in other
residues in the selectivity filter suggests that ion binding does not significantly effect the
chemical environment of the amides in the selectivity filter backbone. This lack of change is likely due to the amides role in maintaining the selectivity filter structure, and since the selectivity filter structure is not affected by ion identity or concentration the local environment of the amides are minimally affected.

Measurement of ion binding in both the selective and non-selective NaK filter would provide the best picture of specific ion binding on ion selectivity, however, the lack of stability of NaK2K without K\(^+\) has prevented this. The lack of stability of NaK2K suggests that in a membrane, the selectivity filter plays a role in stabilizing the structure of the channel. Ion binding to the selectivity filter for other K\(^+\) channels has been shown the stabilize the protein structure\(^{23}\). In light of data from chapter 3, where NaK2K has a more stiff selectivity filter region, a loss of stability in this region would likely have a more major effect on overall protein stability.

**Backbone Dynamics and Selectivity**

The promise of measuring the dynamics of both a selective and non-selective filter is amongst the most exciting directions for the NaK system. As yet, a clear experimental measure of the dynamics in the filter, and how this correlates to selectivity has not been performed. The hetNOE data presented in this chapter shows that these measurements are possible for the NaK system. The data shows that the selectivity filter of the selective NaK2K is fairly stiff as expected. Measurement of the backbone dynamics for the selectivity filter of the non-selective NaK and NaK2K Y55F will help reveal if dynamics play a role in selectivity. This is especially true of the NaK2K Y55F mutant, which maintains a backbone structure identical to the selective filter, but is non-selective. This provides a clean system to characterize whether loss of selectivity occurs
due to changes in dynamics within the four-ion binding site selectivity filter structure. It may also be the case that NaK2K Y55F can change conformations based on ion identity, which would be revealed by spectra of NaK2K Y55F in sodium or low ions. Use of this mutant will help determine the role of ion binding and backbone dynamics on ion selectivity.

References

Figure 1. Ion Binding to the Selectivity Filter of NaK. A. $^{15}$N/$^1$H spectra of NaK at 45°C in low ion conditions (red) and 40mM KCl (black). Selectivity filter residues are labeled. Inset shows titration of Ser70 with increasing concentration of KCl. B. Fitting the ion titration of Ser70 gives a binding constant of 10uM. This fitting is systematically off as the titration is curved. C. Ser70 shown on the NaKΔ19 structure (3EH8)
Figure 2. Allostery in NaK2K Y55F similar to NaK2K. A. $^{15}$N/$^1$H spectra of NaK (black), NaK2K (red) and NaK2K Y55F (blue) at 45°C. Residues discussed in the text are labeled on the spectra. These changes in chemical shift between NaK2K Y55F and NaK (B) and between NaK2K Y55F and NaK2K (C) are mapped on the NaK structure (3E8H).
Figure 3. Picosecond-Nanosecond dynamics in NaK2K. Relaxation due to hetNOE’s are plotted, with a cartoon of the secondary structure of NaK2K labeled below.
Chapter V

Channel Gating Mediated by Interactions with the M0 Helix in the NaK Channel

Joshua Brettmann, Katherine Henzler-Wildman

This chapter contains unpublished work that I have done. I performed and designed the majority of the work presented here. Andrew Meiburg collected a number of the charge mutants presented in this Chapter during his rotation under my supervision. Of note, in this chapter NaK refers to the full-length construct of NaK, unlike previous chapters where NaK referred to NaKΔ19.

Introduction

Ion channels provide a conductive pathway for charged ions to diffuse across the membrane\(^1\). Formation of this path is dependent upon allosteric communication from a regulatory domain to the inner gate of the ion channel\(^2\). Regulatory domains modulate the state of the inner-gate by exerting force on the pore via interactions with the M1 or M2 helix\(^3\)\(^{-8}\). Flexible linkers or small amphipathic helices mediate the interaction, however, the molecular mechanism of this interaction is not fully described. This chapter will examine the mechanism through which the M0 amphipathic helix of NaK changes the state and dynamics of the inner gate of NaK\(^9\)\(^{,10}\). The results of this chapter suggest that the M0 helix plays an important role in regulating the dynamic state of NaK, and that interactions between the M0, M1 and M2 helix are modulated through side chain interactions, not just a physical pull interaction on the backbone (Fig1).
A single regulatory helix on NaK makes it an attractive model system to study the molecular mechanism and dynamics of allosteric regulation of ion channels via solution NMR since the small M0 helix simplifies the system and maintains a size amendable to current solution NMR techniques. Further, NaK has been crystalized both with and without the M0 helix; the removal of this helix leads to an open pore, while the presence of the M0 helix results in a closed pore state.\textsuperscript{9,10} Rearrangements upon channel opening in NaK are similar to what has been observed with other channel structures and have been thoroughly described in Chapter 1. While the structures of the open and closed states of NaK have been solved, the molecular mechanism of the M0 helix stabilizing the closed state has not been described. A molecular description of the communication between the regulatory helix and the inner gate would aid in understanding the method through which regulatory signals are passed to the pore. The generalizable motions involved in gating will allow knowledge learned from NaK to be applied to a wide variety of ion channels.

Investigation of movements involved in the gating would be greatly aided by prior knowledge of stimuli that trigger gating in NaK. However, as this is unknown it is necessary to start the investigation of communication between the M0 helix and the inner gate by examining mutants that change NaK gating. Recently, random mutagenesis by the Perozo Lab at University of Chicago (Personal Communication) identified a number of gain-of-function mutations for NaK. Many of these mutations are found at the hinge or at the bundle crossing; however, one mutation was on the M2 helix between the hinge and bundle crossing facing away from the ion conduction pathway, F94L (Figure 1). This residue is part of a hydrophobic patch that connects residues from the M0, M1 and
M2 helices and rearranges between the open and closed NaK structure, with Phe94 shifting up to interact with Phe28 and moving away from Phe24 (Fig1 A and B). This hydrophobic patch is primed to allosterically transfer a regulatory signal from the M0 helix to the pore. Another interaction that may play a role in the regulation of NaK gating is the nearby salt bridge between Glu23 on the M1 helix and Arg10 on the M0 helix of an adjacent monomer. This chapter will investigate these gain-of-function mutations of NaK, and provide preliminary evidence that this hydrophobic patch and salt bridge play a role in the communication between the M0 helix and the inner gate.

**Results**

**Assignment of NaK**

In previous chapters I showed that NaKΔ19 has remarkably high quality spectra for a membrane protein embedded in isotropic bicelles. In comparison, full-length NaK spectra have significantly more spectral overlap and increased line broadening (which leads to increased collection time) at 45°C. The addition of 19 amino acids in NaK likely contributes to the overlap and increased line broadening (with slower tumbling), however, it is apparent that protein dynamics also play a role in the decreased spectral quality. Spectra of NaK reveal peak doubling for a number of well-resolved peaks. Peak doubling suggests that NaK is either in two isolated states, or is exchanging between two states in the slow exchange regime. Increasing the temperature to 65°C leads to a single set of peaks, suggesting that the peak doubling observed is likely due to dynamic exchange, where raising the temperature increases the rate of exchange or favors a single state (Figure 2A).
To determine if this exchange is localized to regions around the M0 helix or is a global process, NaK spectra must be assigned. This assignment process was aided by assignments from NaKΔ19, of which 83% of peaks were assigned. Spectra of NaK and NaKΔ19 at 65°C overlay nicely allowing for transfer of assignments of many resolved residues (Figure 2B). I assigned NaK, as described in Chapter 2, using $^{15}$N, $^{13}$C, $^1$H labeled samples using Best-TROSY backbone walk experiments and transfer of assignments from NaKΔ19 where possible. This approach led to assignment for 75% of peaks assigned for NaKΔ19 and novel assignment of 14 additional residues on the M0 helix and base of M1 that were not assigned for NaKΔ19.

**NaK Differs from NaKΔ19 Below the Hinge**

Crystal structures suggest that removal of the M0 helix leads to major structural rearrangements, however, most of these changes occur below the hinge (Gly87). Mapping chemical shift changes between the “open” NaKΔ19 and “closed” NaK largely reflects that major changes in chemical shift occur below the hinge (Figure 2C) on the M1 and M2 helix $^{9,10}$. As expected, chemical shifts in the selectivity filter are not sensitive to the addition of the M0 helix. However, some smaller and unexpected chemical shift changes are also seen at the top of the M1 helix (Tyr42, Glu46, Gly47, Arg49) and pore helix (Asn52). The small shifts at the top of M1 and pore helices suggest coupling between the inner gate and selectivity filter region. This analysis was only done with the 65°C spectra, as peak doubling and overlap made transferring assignments at 45°C very limited.

**NaK dynamics are global**
To determine if the peak doubling seen for NaK is global, a peak assignments for NaK at 65°C were transferred to 45°C for well resolved peaks. This gives reporters throughout the protein and shows that NaK is undergoing a global exchange process. As highlighted in Figure 3 A, B, peak doubling is seen at the end of the M0 helix (Trp18) near the hydrophobic patch, which rearranges upon channel opening (Gly93), near the hinge (Ile86) and in the pore helix (Val59) (Figure 3). It is difficult to assess the populations from the peak intensities for all these peaks as spectral quality at 45°C is poor, however, W18 is ~90% one state and ~10% the minor state. These states cannot be assigned to a particular channel state (ex: open or closed), but investigating accessibility via NMR or correlating the populations and rate of exchange with values determined by single channel electrophysiology measurements may facilitate assignment of these states.

**Gain of Function mutations**

Major chemical shifts between NaK and NaKΔ19 occur at the base of the M1 and M2 helix, especially in a hydrophobic patch that links the M0 (Trp18), M1 (Phe24, Phe28), and M2 helix (Phe92, Phe94), which rearranges upon channel opening. Disruption of these aromatics have been shown to modulate either open probability or channel conductance in NaKΔ19 (F92A) 10 or NaK Full-length (F94L) (Eduardo Perozo), however the mechanism through which these gain-of-function mutations work is unknown and will be explored in this chapter. Gain-of-function mutations are a useful tool to both understand functionally important regions of a protein, and relate structural and dynamic changes caused by these mutations to functional consequences.

To measure the extent to which these two gain of function mutations, F92A in NaKΔ19 and F94L in NaK change the structure and dynamics of NaK, 15N, 1H TROSY-
HSQC’s of the two studied gain of function mutants were collected at 45°C and 65°C (Figure 4). NaKΔ19 F92A was described as not significantly changing the structure outside of removing the bulky phenylalanine in the conduction pathway. The TROSY-HSQC supports this finding. Chemical shift changes are largely located in nearby residues (Gly89, Leu90, Gly93, Phe94, Ile95, His96, Leu97) with smaller shifts seen in some residues from Leu82 to Gln103. However, chemical shift changes are very minor outside of nearby residues. No peak doubling or obvious changes in peak intensities suggest that this mutant minimally perturbs the dynamics of NaKΔ19. The localization of these shifts indicates that the structure of the gain of function F92A is likely an opening of the conduction pathway, not a major shift in the state of the gate.

In contrast the gain-of-function mutant, NaK F94L, shows chemical shift changes not just localized to the site of mutation. Mapping the chemical shift changes on the structure of NaK indicates that indeed the largest chemical shift changes are in the immediate vicinity of the mutation, with large shifts in the aromatic pocket (Phe92, Phe28, Phe24, Trp18) and surrounding residues. However, unlike F92A, shifts are not limited to the immediate region. Chemical shift changes are also seen in the hinge, pore helix, loops, and in the selectivity filter (some shifting residues highlighted in Figure 3C). The M0 helix also has limited chemical shift changes localized to the region facing the aromatic pocket (Cys15, Ala19), as well as Arg10, part of the salt-bridge connecting the M0 helix and M1. The extent of these shifts indicates that a more significant structural change is occurring in this gain-of-function mutation than is seen for NaKΔ19 F92A. This is expected, as NaKΔ19 does not need to shift to an open state, it just needs an
increase in conductance, whereas a gain-of-function mutant for NaK would require a shift to an open state.

**Further disruption of the Hydrophobic Patch**

No gain-of-function mutations have been identified that suggest that the M0 helix controls the gating state of NaK. However, looking at the structure of NaK, Phe24 is in a prime position to serve a role in transferring signal from the M0 helix to the inner gate. $^{15}$N, $^1$H TROSY-HSQC’s or NaK F24A were collected at 65°C. Large chemical shift changes between NaK and NaK F24A are seen throughout the hydrophobic pocket (Figure 5 A,B). Interestingly, the chemical shifts of the residues in the hydrophobic pocket resemble the chemical shifts of those residues in NaKΔ19 (Figure 6 A-C). This is clearly seen for Phe28 and Phe94, but also seems to occur for Gly87, Gly89, Ile88, and Val100, where the chemical shifts do not completely overlap but are more similar to the chemical shifts of NaKΔ19 than they are to NaK (Figure 1A,B and Figure 6). Other M2 residues are overlapped in NaK, preventing analysis of all peaks. The M0 helix is also affected by the F24A mutation, as many of the assigned M0 helix residues show chemical shift changes. Residues facing the hydrophobic pocket show the greatest effect, with a large chemical shift changes seen for Trp19, as well as Ala18, and Cys15. Further peak movement is seen in Leu5, Leu8, and Arg10, which suggest that a structural or dynamic shift happens in the M0 helix as well as in the gate.

A structural change toward NaKΔ19 is not the only major change caused by NaK F24A. Significant differences in dynamics are seen for this mutant. Line broadening caused by dynamic exchange is seen in the 45°C spectra of NaK F24A for many residues outside the M0 helix and helical core of the protein (Figure 7). Widespread line
broadening suggests that global dynamic exchange is happening similar to wild type NaK. This is likely due to an altered and faster rate or to an increase in number of states sampled. And like WT NaK, NaK F24A can be shifted to a single state by increasing the temperature. Of particular interest is the Trp18 side chain in NaK F24A, which like wild type NaK shows two states. The minor state is similar to one of the wild type states, while the second state is much further from the chemical shift observed in wild-type NaK. This up field shift in Trp18 suggest this residue is in a more rich electronic environment, but a structural interpretation of this shift cannot be made without more information.

**Charged residues on the M0 helix**

The M0 helix is amphipathic and has a number of charged residues facing away from the membrane, as well as a single arginine (Arg10) involved in a complex hydrogen bond with the adjacent monomers M1 and M2 helix. These charge residues, especially Arg10 seem primed to be involved in regulation of the NaK channel. In order to test this $^{15}$N, $^1$H TROSY-HSQCs were taken of three charge neutralization mutants (R10Q, R13Q, R17Q) as well as combinations of these mutants. Single mutations to the two arginines facing the cytosol (R13Q, R17Q) show limited chemical shift changes or changes in peak doubling, with the chemical shift changes only seen in the unassigned M0 helix (Figure 9). However, mutation to R10Q has drastic chemical shift effects throughout the spectra at both 45°C and 65°C (Figure 8). Beyond the effects on the chemical shift, neutralizing Arg10 changes the dynamic exchange seen at 45°C. This is especially clear for Trp18, as a single peak is seen. Other residues also have changed dynamics, such as a single peak for Val59, Ser70 and Gly93 (Figure 8A). This suggests that exchange between the two
states is either faster, leaving a single average peak between the two states or that a single state is stabilized. While it is difficult to assess if any peak doubling remains in overlapped regions for the R10Q mutant, the lack of peak doubling in areas that had peak doubling for NaK suggest a global shift in dynamics through one of these two methods. Other dynamic shifts are also seen as some peaks (Gly67 and Gly87) have significant line broadening in the R10Q mutant.

Mapping of the chemical shift changes between NaK and NaK R10Q at 65°C show significant chemical shift changes throughout both the M1 and M2 helix (Figure 9E). Of particular interest are chemical shift changes in both the hinge region and the hydrophobic patch, which suggest that removing the salt bridge connecting the M0 helix to the adjoining monomer causes structural and/or dynamic changes throughout the protein. Work in chapter 3 showed that allosteric communication between the selectivity filter and inner gate exists, or is engineered in NaK, and this work further confirms allosteric communication between gates in the opposite direction as mutations in the M0 helix lead to chemical shift changes in the pore helix and the top of the M1 and M2 helices, far from the mutations.

**Similar shifts for R10Q and NaK2K near the hinge**

In both this chapter and previous chapters I have shown allosteric coupling between the selectivity filter region and the inner gate. Unlike the mutation F24A, R10Q does not shift NaK into a state similar to NaKΔ19. However, comparison of NaK2K and NaK R10Q suggests that there may be some similar structural changes shared by the two (Figure 10A-D). Here, we present the spectra of NaK2K in the construct containing the M0 helix, whereas previously we reported the NaK2KΔ19 spectra. Similar to NaK, many
assignments for well resolved peaks can be transferred from the NaK2KΔ19 to the full length NaK2K spectra at 65°C. NaK2K has poor spectral quality at 45°C, limiting analysis to spectra collected at 65°C. This analysis confirms that similar allosteric communication occurs in NaK as was reported in NaKΔ19. Chemical shift changes between NaK and NaK2K are extended into the M0 helix for the full-length proteins, with shifts seen in Trp19, Ala18, Cys15, Arg10.

The chemical shifts of NaK R10Q and NaK2K show some remarkable similarities in comparison to each individually with WT NaK. This is highlighted in (Figure 10) where shifts are seen at the hinge region for both NaK2K and NaK R10Q. For F85 and I84, the shifts in both NaK2K and NaK R10Q are very similar. However, below the hinge NaK2K and NaK R10Q diverge. Further analysis of the dynamics of this system, and these two mutants in particular, may indicate that the structural and dynamic changes in NaK2K are similar to changes induced by NaK R10Q.

**Discussion**

**Allostery in NaK is Related to Dynamics**

This works presents the first NMR experiments done on NaK solubilized in membrane mimicking isotropic bicelles\(^\text{12}\). In contrast to the NaKΔ19, which has a single set of sharp peaks, NaK undergoes conformational exchange on the slow or intermediate timescale. The single set of peaks seen in NaKΔ19 spectra suggests faster dynamics or the preference of a single state, which is likely the reason it is the preferable model system for crystallography. For solution NMR, this dynamic exchange offers a unique opportunity to study the dynamics of channel gating that cannot be done in atomistic
detail with other methods. Global exchange seen for NaK strongly suggests allosteric coupling between the M0 helix and the hinge and gate. Further, exchange in the pore helix suggests that the selectivity filter region is also sensitive to the state of the regulatory M0 helix, which fits nicely with the model of allosteric coupling discussed in Chapter 3. While ideally it would be possible to assign the populations to the open and closed states shown in NaK crystal structures, it is not possible at this point to do that. Continuing this work and determining either structural features of the states via NMR, or identification of populations based on electrophysiology should allow for assignment of the two states.

Long-range effects arising from changes in the M0 helix further supports the idea that allosteric communication is important for NaK function. Chemical shift changes in the hinge, as well as some minor chemical shift changes in the pore helix, were seen for both NaK R10Q and F24A. The large shifts at the hinge region were of particular interest as this region is functionally important as the pivot for channel gating, and likely plays a role in the allosteric communication between the selectivity filter and inner gate during inactivation. The dramatic dynamic effects caused by these mutations show that the study of ion channel dynamics is important for understanding function. NaK F24A increases either the exchange rate into intermediate exchange, or increases the number of states sampled at 45°C as shown by exchange broadening. This exchange broadening is not limited to the M1 and M2 helices, but occurs in the selectivity filter and pore helix. Analysis of the number of subconductance states and their lifetime, as well as the frequency of channel opening, by electrophysiology may help determine if this is caused by an increase in the number of states or a changed rate of channel opening. NaK
R10Q also has changes in dynamics, but instead of exchange broadening, it shifts into a single state or is undergoing fast exchange. This work shows that the structure and dynamics of NaK are tuned by interactions between the M0 helix and the pore, and that changes in this interaction are allosterically transferred throughout the protein. This highlights the necessity of studying ion channel dynamics and how these are tuned by regulatory domains.

**Disruption of M0 Helix Interaction with Pore Changes Channel Structure**

Chemical shifts are useful in determining structural similarities between the mutants analyzed, as these shifts report on the local chemical environment and structure. Comparing the chemical shifts measured here defines similarities in a number of states. Of interest first are the gain-of-function mutants for NaK. These mutants do not cause major structural changes outside of the region surrounding the mutation. This is unsurprising for NaKΔ19 F92A, which has been crystalized and shows minimal changes. A larger effect was expected for the NaK F94L, as Phe94 is involved in interactions in a hydrophobic patch that seem important in tuning the dynamics of the channel as discussed previously. But this mutation again shows limited chemical shift changes. This may be because leucine can fit well within the hydrophobic pocket. As major structural changes do not seem to occur, the gain-of-function is likely caused by the increased flexibility of the M2 helix with the less bulky leucine and in fact a number of subconductances are seen for NaK F94L (Perozo, unpublished data). NMR dynamic experiments would be able to address this hypothesis further. Analysis of how specific mutations in the M2 helix change the dynamics of the M2 helix, and how this change manifests itself in functional changes in channel open probability, conductance, and
number of conductance states may help understand the molecular mechanisms of these properties.

Contrary to Phe94, Phe24 changes both channel dynamics and structure, even though these two residues are located in the same hydrophobic pocket. Comparison of the chemical shifts of NaK, NaKΔ19 and NaKF24A interestingly shows that introduction of the small alanine in the hydrophobic pocket restores residues in this pocket to a structure similar to NaKΔ19, as seen by the similarity of the chemical shifts. The chemical shift similarity between F24A and NaKΔ19 extends beyond adjacent residues, and includes the hinge region. If NaKΔ19 is in the open state as suggested by the crystal structure, this mutation would likely increase the open probability of NaK. This is not surprising as this residue is primed to transfer changes in the M0 helix to the hinge.

If the proposed interaction between Arg10 and the M1 helix does play a role in regulating the state of the channel, shifts similar to F24A would be expected; however, this is not the case. If anything, the chemical shifts of NaK R10Q more closely resemble the chemical shifts of NaK2K, particularly near the hinge. This mutation does cause a global shift of both dynamics and structure, yet without information regarding the state of the hinge and gate in NaK2K it is hard to interpret these results.

References


**Figure 1.** Rearrangements in the Hydrophobic Patch. (A PDB 3E8H) and NaK (B PDB 2AHZ). This interaction involves F24 (green) F28 and F94. Residues that show similar chemical shift to NaKΔ19 for the NaK F24A are highlighted in red. This suggests that F24A is structurally similar to NaKΔ19 in this pocket and elsewhere throughout the M2 helix and gate.
Figure 2. Comparison of NaK and NaKΔ19. Pore helix and selectivity filter are similar with large chemical shift changes below the hinge. A. $^{15}$N/$^1$H spectra of NaK at 45°C (black) and 65°C (red). Peak doubling is seen at 45°C, this becomes a single set of peaks at 65°C. B. Overlay of NaK and NaKΔ19 $^{15}$N/$^1$H spectra at 65°C. C. The chemical shift difference between NaK and NaKΔ19 at 65°C mapped on the NaKΔ19 structure (PDB 3E8H). There are shifts throughout the spectra, but the largest shifts are localized to below the hinge. This matches what is expected based off the crystal structures of NaK and NaKΔ19.
Figure 3. Global Exchange in NaK. Residues throughout NaK show peak doubling. Residues are highlighted in expanded regions. These residues are mapped on the NaK structure (PDB 2AHZ) and indicate exchange is seen in the M0, M1, pore and M2 helix.
Figure 4. Coupling between the M2 Helix and M0 Helix is mediated through a hydrophobic patch. $^{15}$N/$^1$H spectra of NaK (black) and NaK F94L (red) at 45°C (A) and 65°C (B). Highlights of shifts throughout the M2 and M0 helix are shown in C. The chemical shift changes are mapped onto the NaK structure (PDB 3E8H) in a blue to red heat map. Clear chemical shift changes are seen but assignment could not be transferred to the F94L spectra are colored orange. Chemical shift are seen throughout NaK structure. The large shifts in the M0 helix suggest coupling between the helix and pore is mediated through the hydrophobic patch.
Figure 5. Chemical shift changes in F24A in the M0 and M2 helices. A. $^{15}$N/$^1$H spectra of NaK (black) and NaK F24A (pink) at 65°C. Residues referred to in text are labeled. B. Highlighting changes in the M0 helix. The large chemical shift changes to residues in the helix suggest an important role in coupling the M0 helix to the pore. There is a very large shift for W18 side chain, which stacks with F24 in the NaK structure.
Figure 6. NaK F24A similar for NaKΔ19 in the Hydrophobic Patch and the Hinge. A. $^{15}$N/$^1$H spectra of NaK (black) NaKΔ19 (blue) and NaF24A (pink) at 65°C. B,C. Residues of interest discussed in the main text that show similarities in chemical shift of NaK F24A and NaKΔ19. The residues that shift towards NaKΔ19 are highlighted in red on Figure 1. These shifts suggest that NaK F24A resets to a state similar to the open NaKΔ19 state.
**Figure 7.** Exchange Broadening of Peaks for NaK F24A. $^{15}\text{N}/^{1}\text{H}$ spectra of NaK (black) and NaK F24A (pink) at 45°C show very poor spectral quality with significant line broadening. Further, many peaks have disappeared, likely due to intermediate exchange or too many conformational states.
**Figure 8.** NaK R10Q has Significant Chemical Shift Changes and Dynamic Changes Compared to NaK. $^{15}$N/$^1$H spectra of NaK (black) and NaK R10Q (green) at 45°C (A) and 65°C (B). There are many chemical shift changes for NaK R10Q. Also, there appears to be less peak doubling that what is seen for NaK at 45°C.
Figure 9. Arg10 Couples the M0 Helix to Pore. **A,C.** $^{15}$N/$^{1}$H spectra of other charge reversal on the M0 helix (R13Q, orange R17Q, Blue). These mutants do not show major shifts at the hinge. **B,D.** $^{15}$N/$^{1}$H spectra of charge reversal mutants that contain the R10Q mutation all show similar shifts at the hinge (R10Q, green R10,13Q, pink R10,13,17Q, mauve). **E.** Large chemical shift changes between NaK and NaK R10Q mapped on the NaK structure (PDB 3E8H). Residues that shift are red, ones that do not are blue. Residues that assignments were not known or had significant overlap are grey. This indicates that the M0 helix is allosterically coupled to the hinge and gate through Arg10.
Figure 10. Similarities of R10Q and NaK2K at the Hinge. A, B, C. $^{15}$N/$^1$H spectra of NaK (black) NaK2K (red) and NaK R10Q (green) at 65° C. Residues discussed in the hinge region that were discussed in the text are labeled. D. Sites of mutation for NaK2K (red) and R10Q (green) are shown. Residues in R10Q that have similar chemical shift to NaK2K are highlighted in orange. Residues with different chemical shifts in all three spectra are labeled in blue. The similarity of shifts near the hinge region, far from the site of either mutation suggest that similar allostery could be occurring from the M0 helix to the hinge as occurs between the selectivity filter and hinge.
Chapter VI

Conclusion and Future Directions

Optimization of Expression and Solubilization for NaK

This thesis work presents the initial characterization of NaK for solution NMR studies. Prior to this work, NaK had been used to study ion selectivity with crystallography, ITC and computation simulations\(^1\)-\(^{14}\). My work shows that NaK is an excellent system to study with solution NMR, and that NaK can be an excellent system to not just study ion selectivity, but also allostery between gates, which is present in many ion channels. While studies of membrane proteins with solution NMR are increasing, these studies are not trivial\(^15,16\). The large size of these systems and difficulty in finding a lipid-mimicking environment has hindered their study. Spectral overlap was long thought to be a major problem in membrane protein NMR, but this work, combined with study of other membrane proteins, suggests that well-folded and packed membrane proteins give well-dispersed peaks, suitable for a wide range of NMR studies\(^17,18\).

Work in this study also supports the use of isotropic bicelles as an excellent membrane mimetic environment to study membrane protein by NMR\(^19\). The NaK spectra shown in this work are higher quality than published spectra for KcsA, which were solubilized in detergent (Chapter 3 Figure 2). Further, spectral quality of NaK in DM, a common detergent used for crystallography and NMR, was much lower quality than spectral quality obtained from bicelles. This shows that a good membrane mimetic is necessary for solution NMR work on membrane proteins. Isotropic bicelles are also able to incorporate a wide array of long chain lipids, allowing for study of specific protein-lipid interactions\(^19\). Lipid-specific interactions are important for a wide variety of
ion channels, such as PIP2 and cholesterol for Kir channels\textsuperscript{20,21}. NaK is insensitive to many changes in membrane composition, giving identical spectra when solubilized with POPE:POPG, and with small amounts of PIP2. However, when loaded into a bicelle with 70:20:10 POPG:POPE:Cardiolipin as the long chain lipid NaK shows significant chemical shift changes, as well as changes in dynamics, as seen by peak doubling for Trp18 (Figure 1). Work in this study did not further pursue lipid specific effects for NaK, but this line of study should be pursued as cardiolipin has been shown to be an important regulator for KcsA\textsuperscript{22,23}, and this change in dynamics could be the mechanism by which the regulation occurs. Work investigating the structural and dynamic effects of specific NaK lipid interaction can help understand lipid regulation of other ion channels.

**Assignment of NaK**

This work provides the backbone assignments of NaK, a crucial step in interpreting NMR data. Assignment of helical membrane proteins is not trivial, especially in systems that cannot fully exchange deuterons for protons in the amide backbone. Using new NMR technologies, such as BEST-type pulse sequences combined with non-uniform sampling, allowed for a high degree of assignment for NaK\textsuperscript{24–27}. This strategy can be used for the assignment of spectra of other membrane proteins. The assignment for NaK\textsubscript{Δ19} was 85% complete for the backbone and these assignments were deposited to the protein databank. Assignment for NaK\textsubscript{2K} and NaK were not fully completed, and follow-up work to complete these assignments are necessary.
**Allostery in NaK**

This thesis work identified unexpected allostery between the selectivity filter and inner gate of NaK. This was shown by chemical shift changes throughout the NaKΔ19 and NaK structures when a K⁺ selective filter was introduced (Chapter 3 Figure 4). Crystal structures of this mutation were nearly identical (0.24Å all atom RMSD) outside the immediate region of mutation (Chapter 3 Fig1) 5, which suggests that the chemical shift changes should be located only in the selectivity filter region. This chemical shift change far from the site of mutation is likely caused through an allosteric change in NaK below the hinge. The allosteric change is mediated by formation of a four-site selectivity filter (as identified in crystal structures). Individual point mutants that create NaK2K do not show the allosteric changes, but the four-site non-selective NaK2K Y55F has similar allosteric changes below the hinge as what is seen for NaK2K (Chapter 3 Fig 4 and Chapter 4 Fig3). A number of other NaK selectivity filter mutants have been crystalized, including filters with three ion-binding sites 4. Future work should determine if these filters also show allosteric changes. Analysis of peaks intensities for NaK and NaK2K show NaK2K has a stiff selectivity filter region, with a correspondingly less rigid hinge and gate, suggesting that these allosteric changes are mediated by changes in protein dynamics.

Allostery between gates is important for gating at the selectivity filter in C-type inactivation for KcsA and many eukaryotic channels, but no evidence of this allostery had been shown for NaK prior to this work 28–31. Further, there is no evidence for selectivity filter collapse for NaK; however, this is in part due to lack of electrophysiological experiments. If gating occurs at the selectivity filter for NaK, it is
unlikely to be structurally similar to KcsA, the current structural model for C-type inactivation\(^{28,29,31}\). Structural changes for inactivation must be different for NaK, as the selectivity filter is the main structural deviation between NaK and KcsA; however, it is also clear that the general mechanism is likely different because the selectivity filter of NaK does not collapse in low ion conditions (Chapter 3 Fig3), as occurs in KcsA\(^{28,29,31}\). The structural collapse of the KcsA filter is thought to be a necessary step in the allosteric coupling between the gates\(^{28,29,31,32}\). However, NaK still exhibits allostery between the selectivity filter and inner gate without the structural collapse. This suggests that allosteric linkage may not be dependent upon selectivity filter collapse, but rather is a more general interaction between the gate and selectivity filter. Further, it suggests that allostery between gates is likely important for a wide variety of channels beyond K\(^+\) channels\(^{31,33–36}\).

It also provides evidence that the collapsed state of the KcsA filter may not be a good model for C-type inactivation\(^37\). The base of the selectivity filter and pore helix of NaK resemble KcsA, and it is likely that this region is important for the allosteric connection\(^38\). Dynamic exchange shown in the CPMG experiments at the base of the selectivity filter and hinge region suggests these regions may be dynamically coupled (Chapter 3 Figure 6). This model would be strengthened by electrophysiological measurements that will suggest the number of exchanging states for NaK, allowing dynamic measurements to be tied back to channel functions. This will allow us to determine whether the motions in the selectivity filter base and hinge are related to the same process.
Similar to NaK, selectivity filter collapse and inactivation is not expected for NaK2K, as crystal structures of NaK2K without K$^+$ show no change in selectivity filter structure\(^8\). Interestingly, unlike NaK, NaK2K is unstable in bicelles in the absence of K$^+$. My work did not examine the concentration of K$^+$ needed to stabilize NaK2K, or whether other ions are able to stabilize the filter. But the lack of stability of NaK2K in the absence of K$^+$ suggests that ions in the selectivity filter are needed to stabilize the filter, and that loss of stability in the filter may lead to loss of protein stability. Both KcsA and KirBac1.1 have differential stability depending on ion concentration or identity, which may be the case for NaK2K\(^{39}\). Further work looking at how ions affect NaK2K stability will help determine the energetic cost of maintaining a selective selectivity filter. Further, it would be interesting to use the four-site non-selective NaK2K Y55F to test whether a possibly less-rigid selectivity filter is stable without ions.

**M0 Helix and Inner Gate Allostery**

Spectra of NaK indicate a major shift in dynamics compared to NaKΔ19. NaK has significant peak doubling throughout the NaK structure indicative of global exchange (Chapter 5 Figure 2,3). This is likely due to dynamic exchange between states, as increasing the temperature leads to a single set of peaks. This increased dynamics for NaK is likely a reason that NaKΔ19 is the construct of choice for crystallography. The global exchange of NaK is the first evidence in this system that the allostery works both from the selectivity filter to the gate and from the regulatory domain to the hinge. Similar peak doubling at the base of the pore helix (Val59) and the hinge (Ile86) supports
a model where allostery is mediated by pore helix-M2 helix interactions. Future work on this system should pursue measurement of the dynamic exchange in NaK.

Differences in chemical shifts between NaK and NaKΔ19 at 65°C, the temperature at which NaK exists in a single state, are largely consistent with the expected structural change shown in crystal structures\textsuperscript{3,5}. The largest changes are seen below the filter in the hinge and gate region (Chapter 5 Figure 2), which rearrange between open (NaKΔ19) and closed (NaK) states (Chapter 1 Figure 3). It is difficult to compare chemical shifts at 45°C due to peak doubling in NaK, but this analysis does not support a model where NaK is shifting between the closed state and the open state, because the chemical shifts of the minor peaks do not overlay with NaKΔ19 (the open state NaK). However, it is possible that the addition of the M0 helix in the full-length protein puts constraints on the pore in the open state, changing the structure or dynamics of the protein, and that the minor peaks in fact represent this constrained open state.

Investigation of the dynamics and structure of NaK and NaKΔ19 would help identify allosteric connections between the regulatory domain and hinge. Likely experiments would include RDC measurements to assess helix orientation and PRE-accessibility to determine the state of the inner gate.

This work also probed specific interactions between the M0 helix and pore domain. M0 helix and pore interactions are likely mediated through side chain interactions, with a complex salt bridge serving as an inter-monomer connection at one end of the pore helix, and an aromatic cluster working as an intra-monomer link (Chapter 1 Figure 4). Hydrophobic and charge interactions have been shown to link regulatory domains to the pore through an interfacial helix for the eukaryotic channels Kv and Kir,
respectively\textsuperscript{40–44}. Similar to charged interactions in Kir, charge neutralization of Arg10 in NaK led to both dynamic and chemical shift changes in NaK (Chapter 5 Figure 8). Interestingly, the chemical shifts at the hinge for NaK R10Q resemble chemical shifts for NaK2K; however, the structural and/or functional interpretation of this is not clear (Chapter 5 Figure 10).

Removal of a Phe24 (F24A) located in the intra-monomer hydrophobic patch also leads to large chemical shift and dynamic changes (Chapter 5 Figure 5, 7). The chemical shift changes show that Phe24 is necessary to maintain a closed channel state, as the chemical shift of this mutant is very similar to NaKΔ19 at the hinge (Chapter 5 Figure 1, 6). Together these two mutants strongly suggest that the M0 helix allosterically regulates the state of the inner gate through side chain interactions on both ends of the helix. However, at this point it is unknown how these mutations affect the functionality of NaK, and functional measurements should be done. This regulation is novel, as slide helices from other channels have a single site of interaction. However, investigation of how the M0 helix can allosterically communicate with the gate and selectivity filter will help understand regulation through interfacial helices.

\textbf{Ion Selectivity}

The initial goal of this project was to measure the contribution of selectivity filter dynamics to ion selectivity; however, unexpected allostery became the main focus of my work. Preliminary data suggests that solution NMR with NaK can contribute to our knowledge on the molecular mechanism of ion selectivity. Measuring both ion-protein interactions through chemical shift changes in the selectivity filter, and fast timescale
dynamics in the selectivity filter will allow for a more complete description of ion selectivity (Chapter 4 Figure 1,2). To this end, the fast dynamics of the filter of NaK and NaK2K should be compared. More interestingly, direct comparison of structurally homologous filters that have different selectivity, such as NaK2K and NaK2K Y55F, will allow for a clean comparison of the role that dynamics play in selectivity.

**Final Thoughts**

Work performed in this thesis has advanced a new model system to study dynamic features of ion channels via solution NMR. Assignments of NaK and NaKΔ19 allow for a wide range of dynamic studies in the future. This work also showed that there is a tight allosteric coupling between the selectivity filter and inner gate that was unexpected from crystal structure data. This unexpected allostery highlights the importance of studying ion channels both structure and dynamics. It also suggests that allosteric coupling is an inherent feature of ion channel function. Finally, this work lays groundwork for studying the role of protein dynamics in ion selectivity.

**References**


Figure 1. Major Spectral Changes for NaK in 70:20:10 POPG:POPE:Cardiolipin. $^{15}$N-$^1$H TROSY-HSQC of NaK in DMPC Bicelle (black) and NaK in 70:20:10 POPG:POPE:Cardiolipin bicelles (red) at 65°C. NaK in the presences of cardiolipin shows drastic changes in chemical shift. There is major exchange broadening and peak doubling seen for Trp18.
Appendix I

Solution NMR of Monomeric BK Pore Domain

The “Big Potassium”-type potassium channel (BK channel), named for its large conductance is a widely expressed ion channel with a crucial role in cell excitability. This potassium channel is Ca\(^{2+}\)- and voltage-activated, and mutation in this protein have been associated with numerous diseases, making it a therapeutic target\(^1\). Structural studies aid in the development of therapies, but as of yet there is limited structural information about the BK pore domain. It is common to use the structure of other K\(^{+}\) pores as a model for the BK pore, and while this strategy yields useful information, there is evidence of significant differences between the BK pore and other K\(^{+}\) channel pores. My work attempts to use pore-only BK constructs to study structural features of the BK monomer with solution NMR. Unfortunately, a functional pore only construct of BK was not able to be purified. However, studies on the BK monomer can identify unique structural features of the BK channel.

Introduction

General Structure of BK channels.

The BK channel is a tetramer comprised of three structurally separate domains coded in the slo1 gene (Chapter 1 Figure 1). Within the transmembrane region it contains a voltage-sensing domain (VSD, S1-S4) homologous to other voltage-gated K\(^{+}\) channels (Kv channels) and a pore gate domain (PGD, S5-pore loop-S6). Following the PGD is the C-terminal intracellular domain (CTD) which consists of two calcium-sensing domains (Rck domains)\(^2\). The BK channel contains an extra helix prior to the voltage-
sensing domain that is found only in the BK family of Kv channels, however the exact function of this helix is not well understood. While a lot of work has been done to understand the regulation of the BK channel by both the transmembrane VSD and CTD calcium sensing domains, no work has been done looking at intrinsic gating properties of the PGD domain in BK channels. A pore domain only construct could shed light on this question.

Structural studies have been completed on other K⁺ channels. The first crystal structure of an integral membrane protein was Rod MacKinnon’s structure of the bacterial K⁺ channel, KcsA. Since then, several additional K⁺ channel structures have been solved and all have a very similar PGD structure. Thus this structure has been used to model many other K⁺ channel PGDs for which there is no structural data, including the BK channel.

**Significant structural differences between BK and other K⁺ channels exist.**

Recent evidence has suggested that, while the KcsA structure provides an accurate model for many K⁺ channels, for the BK channel, there may be significant differences that warrant further study. Experiments performed by Chris Lingle’s lab have highlighted many of the structural differences between the BK pore and other K⁺ channel pores. First, cysteine scanning studies showed that BK’s pore lining residues differed from what is predicted by the KcsA structure. Second, cysteine modification experiments showed that the BK pore region following the selectivity filter is significantly larger than what is found in other K⁺ channels. Third, the BK channel has a novel interaction with an inactivating β subunit that is stereospecific, unlike other K⁺ channels. Fourth, the BK PGD has novel interactions with pore blockers, especially
shown by the closed-state-dependent blocker paxilline\textsuperscript{9}. This evidence provides motivation for more detailed structural studies of the BK PGD.

Sequence alignment of BK with other K\textsuperscript{+} channels suggests regions of BK that may cause these structural differences. The BK channel has a di-glycine at the conserved glycine hinge, unlike other K\textsuperscript{+} channels. The BK PGD also has an YVP sequence near the cytosolic gate rather than the PVP found in many Kv channels. Both of these regions may be contribute to structural differences between BK PGD and other Kv channels, and will guide this structural study of the BK PGD.

**Results**

**Purification of BK Pore Gate Domain for Solution NMR Studies.**

This work attempted to purify a fully functional BK pore domain. Instead, large quantities of monomeric BK pore (termed BK143) were purified in DPC12 following a similar prep as described in the methods of this thesis. Protein is expressed in *E. coli* in minimal media and is purified using DPC12. This yields a highly uniform monomeric protein (higher order oligomers can be seen when cysteine crosslinking occurs). An example of the prep purity and size-exclusion profile can be seen in figure 1A,B. This monomeric protein was isotropically labeled and a \textsuperscript{1}H/\textsuperscript{15}N TROSY-HSQC was collected (Figure 2). This spectrum showed significant overlap, but the peak dispersion indicates that it is not fully disordered.

Many attempts were made to purify a fully functional BK pore domain. Attempts to refold this protein with numerous detergent/detergent:lipid mixtures have been tried with no success. As KcsA has been refolded from its monomeric form into liposomes, this was also attempted with many lipid composition, since KcsA refolding is lipid-
dependent. The refolded protein was cross-linked with 0.06% gluteraldehyde (Gta) and oligomerization was checked on an SDS-page gel (Figure 3). The presence of bands for multiple oligomeric states, including smears for higher ordered species, indicate that lipids do not stabilize a folded tetrameric BK pore domain. Further, there did not seem to be any indication of specific lipid effects.

An assay exhibiting functionality would be much more effective in determining if this protein is functional. In order to test functionality in liposomes, a rubidium flux assay was done with BK143. BK143 did not show any flux in this type of liposome flux assay (Figure 4). This shows that BK143 is not functional.

**Constructs to recover functional pore**

To try to purify a functional pore, over 100 constructs have been tested for rescue of tetrameric stability and function, this is summarized in the table below.

<table>
<thead>
<tr>
<th>BK construct</th>
<th>Growth</th>
<th>Expression</th>
<th>Oligomeric state</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lengths</td>
<td>Generally good growing</td>
<td>High</td>
<td>Generally monomer in DPC 12. Some odd higher order species with certain constructs. No purification in DM or DDM</td>
<td>Most promising class of constructs</td>
</tr>
<tr>
<td>Forced Dimers and tetramers</td>
<td>Poor Growth</td>
<td>Low expression</td>
<td>Bands of monomer:dimer:tetramer indication of proteolysis. Not good in DM or DDM</td>
<td>Some promise but not further pursuit planned</td>
</tr>
<tr>
<td>Leucine Zipper</td>
<td>Poor growth</td>
<td>Low Expression</td>
<td>No hint of oligomeric state</td>
<td>Not worth pursuing</td>
</tr>
<tr>
<td>Chimeras</td>
<td>Mixed growth, more good than bad</td>
<td>Mixed expression</td>
<td>PoHX and SF chimeras have promise</td>
<td>Small chimerias might work</td>
</tr>
</tbody>
</table>
The BK constructs tested are separated into 4 major classes. Two classes, forced oligomers and leucine zippers, have shown little to no promise in recovering BK pore domain refolding. The forced dimers and tetramers do purify as oligomers, however, they have poor expression and easily degrade. The leucine zippers purify as monomers with very low yield. Both of these strategies have proved insufficient.

Making chimeras of BK pore and bacterial homologue KcsA was another strategy pursued. Regions of BK were put into a KcsA background and protein overexpression and tetramerization were tested. In general it was found that KcsA is not very tolerant to additions of BK regions. One such example of small changes to KcsA that make a big difference in protein stability is found in the selectivity filter. If changes are made just prior to the selectivity filter of KcsA to make it BK-like, no changes in tetrameric changes are seen. If changes are made just after the selectivity filter of KcsA to make it BK-like, tetrameric stability is lost (Figure 5).

This example exemplifies the problems of the chimeric approach to this project. Many different KcsA-BK chimeras could be made, but they would all have to be screened for tetrameric stability. After this screening they would all then have to be screened to see if they maintain the unique BK characteristics we are interested in. So while this strategy might work in the long run, it was not feasible during my graduate career.

**Assignment of BK monomer**

While work with fully functional BK PGD would be ideal, investigation on the monomeric BK could map the secondary structure of the M2 helix. This can identify if there is a unique sharp kink in the M2 helix. To this end backbone walk experiments
were collects for the BK143 protein. Since backbone carbon chemical shifts inform on the secondary structure of the protein assigning the backbone of BK will determine if BK143 maintains a proper secondary structure. Further, deviation from expected alpha helical chemical shift will support a large kink in the M2 helix. To start the assignment process I collected backbone walk experiments that were described in Chapter 2. An HNCA, HN(Co)Ca, and HNCA were collected. The spectral quality of these spectra were promising. Many peaks that were expected to be seen were in the 2D HN projection of the HNCA. However, there is severe spectral overlap, as the monomeric form of this protein does not have the helical packing helps disperse the peaks for NaK (Figure 6). Full backbone assignment of BK143 will be possible with a high degree of deuteration and high field strength to narrow line widths. Further, amino acid specific labeling will provide anchor points for this assignment. If assignments suggest the proper secondary structure, further structural and dynamics questions can be pursued.

Reference


Figure 1: Purification of BK143 in DPC12. A. The size exclusion profile of BK pore gate domain (228-371) also called BK143 and KcsA. KcsA is a tetramer is DPC12, while BK143 is monomeric as seen by the leftward shift of the elution profile. B. SDS-page gel of BK143 and KcsA purified in DPC12. BK runs as a monomer and KcsA runs as a tetramer.
Figure 2. $^{15}\text{N}/^{1}\text{H}$ TROSY-HSQC of BK 143 in DPC12. This spectrum shows that there is significant spectral overlap for this construct, however, the peak dispersion suggests it has some degree of folding.
Figure 3: BK143 Reconstituted into Liposomes. Gta crosslinking on BK143 loaded into liposomes of various lipid composition. BK143 was put into 3:1 POPE:POPG, POPE:POPS and brain extract lipids at ~100 fold excess lipids. Crosslinking was done on BK143 loaded into liposomes using 0.06%GTA.
Figure 4- BK143 loaded into 1:3 POPE:POPG (green) liposomes does not show any significant flux as compared to empty liposomes (black).
Figure 6: 3D-HNCA and HN(co)CA of BK143. Top: 2D projection (N/H) of the HNCA of BK143 (red) overlaid on the 2D BK143 spectra. This indicates that there is signal for a good portion of the BK143 through the backbone. Bottom: 2D projection (C/H) of the HNCA (red) of BK143 and HN(co)CA (blue). Again there is strong signal, with some identifiable i i-1 pairs. However, the overlap will make the backbone walk difficult.