Determinants of cation transport selectivity: Equilibrium binding and transport kinetics

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The crystal structures of channels and transporters reveal the chemical nature of ion-binding sites and, thereby, constrain mechanistic models for their transport processes. However, these structures, in and of themselves, do not reveal equilibrium selectivity or transport preferences, which can be discerned only from various functional assays. In this Review, I explore the relationship between cation transport protein structures, equilibrium binding measurements, and ion transport selectivity. The primary focus is on K+-selective channels and nonselective cation channels because they have been extensively studied both functionally and structurally, but the principles discussed are relevant to other transport proteins and molecules.

Ion transport proteins, including channels, exchangers, and pumps, facilitate the selective transfer of ions across biological membranes (Hille, 2001). Ion channels transport their substrates by electrodiffusion at rates that approach $10^8$ s$^{-1}$, whereas exchangers and pumps are orders of magnitude slower because of the obligatory coupling of the transport event to a conformational change. Crystal structures of transport proteins reveal the arrangement of atoms that directly interact with transported ions and are used to develop detailed models for the structural basis for selectivity. The mechanisms by which these proteins achieve transport selectivity are defined by their three-dimensional structure and can depend on both kinetic and thermodynamic factors. But inferring function from structures can be tricky, in part because transport molecules often adopt slightly different conformations depending on the nature of the bound ligand. For example, the small molecules valinomycin and nonactin readily yield crystal structures bound to Na$^+$ or K$^+$ ions with coordination chemistry typical for the bound ions (Kilbourn et al., 1967; Dobler and Phizackerley, 1974; Neupertlaves and Dobler, 1975; Steinauf et al., 1982). By simply comparing these structures, one might conclude that these small molecules are nonselective. However, transport assays, equilibrium ion-binding assays, and computational studies reveal a substantial K$^+$ selectivity for these small molecules highlighting the importance of integrating information from a three-dimensional structure with experimentally determined ion selectivity measurements (Moore and Pressman, 1964; Graven et al., 1966; Izatt et al., 1985; Eisenman and Alvarez, 1992).

Caution must also be exercised when comparing equilibrium properties of transport proteins that move ions at vastly different rates, as equilibrium properties do not always predict transport selectivity. In the case of exchangers and pumps, hereafter referred to as “transporters,” the active sites tend to be buried in the middle of the protein, and accessible to only one side of the membrane at a time. The obligatory conformational change required to transport the ion (and any other substrate) limits the transport rate (Dutzler et al., 2002; Yamashita et al., 2005; Boudker et al., 2007; Weyand et al., 2008; Gao et al., 2009; Ressl et al., 2009; Liao et al., 2012; Nyblom et al., 2013; Zhou et al., 2014). Therefore, transporter selectivity could be determined by the equilibrium-binding preference for ions alone, which can be experimentally determined with a binding assay and rationalized from co-structures of the transporter with ion (Boudker et al., 2007; Picollo et al., 2009; Reyes et al., 2013).

Ion channels, on the other hand, feature an ion-conduction pathway that spans the protein in at least one conformation (called the open conformation) and contains a series of selective ion-binding sites (Hille, 2001; Jiang et al., 2002; Zimmermann and Dutzler, 2011; Bacoguis and Gouaux, 2012). Although ions moving by electrodifussion through the channel encounter energy barriers, these barriers are rapidly surmounted with thermal fluctuations, and ion channels can sometimes conduct at rates that are near diffusion limited. So, unlike transporters where transport is slow relative to the time it takes to equilibrate a binding site, channels can transport ions more rapidly than the ion-binding sites equilibrate.

Ion channel selectivity in a lipid membrane is typically quantified using one of two operational definitions...
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The functional/structural units of the transmembrane pore that conducts ions, and the regulatory domains that determine if the channel is open or closed (Fig. 1) (Hille, 2001). The regulatory domains include transmembrane voltage sensors, Ca²⁺-binding domains, PAS (Per, ARNT, Sim) domains, Kir cytoplasmic domains, and cyclic nucleotide domains, which are tethered to the cytoplasmic portion of the transmembrane pore domain. The eight transmembrane helices of the pore domain form an inverted teepee, with each of the four subunits contributing two helices. In the open ion-conducting conformation, the intracellular region of the helices is splayed open to reveal a water-filled cavity that can accommodate a hydrated ion. Both electrophysiology and crystallography reveal a modest five- to sevenfold equilibrium selectivity for K⁺ over Na⁺ in the cavity, which may result from the different hydration radius of the two ions and the space available in the cavity (Neyton and Miller, 1988; Zhou and MacKinnon, 2004).

The cavity ion is dehydrated as it enters the channel’s selectivity filter, the portion of the channel responsible for its high selectivity. The selectivity filter (sites S1–S4) is 12 Å long and decorated with evenly spaced oxygen atoms contributed from the backbone carbonyls and hydroxyls from threonine amino acids (Fig. 2). This arrangement of oxygen atoms is found in all K⁺-selective channels studied to date, suggesting a strong evolutionary constraint on this region of the channel (Morais-Cabral et al., 2001; Zhou et al., 2001; Jiang et al., 2003; Kuo et al., 2003; Long et al., 2005, 2007; Nishida et al., 2007; Tao et al., 2009; Uysal et al., 2009; Clarke et al., 2010; Mari et al., 2011; Whorton and MacKinnon, 2011; Brohawn et al., 2012; Miller and Long, 2012). The distribution of electron density in the selectivity filter differs depending on whether the structure was solved in the presence of K⁺ ions, Na⁺ ions, or a mixture of ions, indicating that the K⁺ and Na⁺ ions interact with the channel differently within this region. In both the Streptomyces lividans KcsA channel and the Methanobacterium thermoautotrophicum MthK channel, electron density corresponding to K⁺ ions (or water

Below, I discuss functional, structural, and computational studies of K⁺ channels and some nonselective channels, with the goal of connecting their equilibrium ion-binding properties to their structures and selective ion conduction.

Structures of K⁺-selective channels
The diversity of available K⁺ channel crystal structures has facilitated our understanding of the molecular details of ion channel function (Doyle et al., 1998; Morais-Cabral et al., 2001; Zhou et al., 2001; Jiang et al., 2002, 2003; Kuo et al., 2003; Long et al., 2005, 2007; Nishida et al., 2007; Tao et al., 2009; Uysal et al., 2009; Clarke et al., 2010; Mari et al., 2011; Whorton and MacKinnon, 2011; Brohawn et al., 2012; Miller and Long, 2012). These channels can be roughly divided into two functional/structural units: the transmembrane pore that conducts ions, and the regulatory domains that determine if the channel is open or closed (Fig. 1) (Hille, 2001).
in the presence of Na\(^+\) ions is likely to represent the ducts Na\(^+\) ions in the absence of K\(^+\), its selectivity filter

cances and other indirect methods. Because MthK con-

\[ \Delta G = \left( \Delta G_{channel}^{K^+} - \Delta G_{solvent}^{K^+} \right) - \left( \Delta G_{channel}^{Na^+} - \Delta G_{solvent}^{Na^+} \right), \]

where \( \Delta G < 0 \) is K\(^+\) selective, \( \Delta G > 0 \) is Na\(^+\) selective, and \( \Delta G = 0 \) is nonselective.

K\(^+\) channels have an equilibrium preference for K\(^+\) ions over Na\(^+\) ions in their selectivity filter. Electrophysi-

ology experiments using Ba\(^{2+}\) to block several channels demonstrated a >1,000-fold equilibrium preference for K\(^+\) ions in the extracellular “lock-in” sites with K\(^+\) affinities of \( \sim 20 \) \( \mu M\) (Neyton and Miller, 1988; Piasta et al., 2011). In KcsA, MthK, and NaK2K channels, low concen-

trations of K\(^+\) outcompete high concentrations of Na\(^+\) ions for binding to the filter, based on x-ray crystallo-

graphy (Morais-Cabral et al., 2001; Zhou et al., 2001; Zhou and MacKinnon, 2003; Thompson et al., 2009; Sauer et al., 2013). This qualitative result demonstrates that the selective ion-binding sites are indeed within the selectivity filter. Titrations in both solution and solid-

state nuclear magnetic resonance shows a K\(^+\)-dependent change in chemical shift of atoms in the selectivity filter (Bhate et al., 2010; Imai et al., 2010). Free energy calcula-

tions and atomistic molecular dynamics (MD) simulations also demonstrate an equilibrium preference for K\(^+\) ions, even when Na\(^+\) ions are not restricted to the center of observed K\(^+\) sites (Luzhkov and Aqvist, 2001; Noskov and Roux, 2006; Varma and Rempe, 2007; Egvold and Roux, 2010; Kim and Allen, 2011). And fi-

nally, equilibrium ion binding to several K\(^+\) channels was measured using isothermal titration calorimetry (ITC) (Lockless et al., 2007; Liu et al., 2012; Liu and Lockless, 2013). The observed K\(^+\) affinities are in the range of 10–100 \( \mu M\), which is consistent with electrophysiology measurements of the \( k_{off} \) rate of \( 10^3 \) s\(^{-1}\) for the last ion leaving the filter of Shaker channel (if one assumes the diffusion-limited \( k_{on} \) rate of \( 10^9 \) M\(^{-1}\) s\(^{-1}\); \( k_{off} = k_{off} / k_{on} \) of \( \sim 10 \) \( \mu M\)) (Baukrowitz and Yellen, 1996).

Several mechanistic explanations for the equilibrium selectivity have been proposed based on MD simulations and free energy calculations. On the one hand, Roux and colleagues proposed from MD simulations that K\(^+\) selectivity arises from a field strength match between
K+ channels have equilibrium preference for K+ ions and the charge density of K+ ions, which is different from that of the smaller Na+ ions (Roux et al., 2011). This conclusion was based on both a model ion-binding site mimic of site S2 of KcsA, where coordinating dipoles are free to move in a defined area of the structure, and on simulations of the multi-ion free energy landscape of a K+ channel, where a greater selectivity for carbonyl oxygen atoms versus water molecules is observed (Noskov et al., 2004; Egwolf and Roux, 2010). Rempe and colleagues, on the other hand, argued that chemistry is important, but not the driving force in selectivity of K+ channels. Using free energy calculations with density functional theory, they found selectivity arises from an environment that imposes constraints on ion-binding sites (Varma et al., 2008, 2011). This topological control emerges from intramolecular hydrogen bonds within the ion-binding molecule to constrain the number of oxygen atoms that coordinate the ions, leading to over-coordination. Further experiments are necessary to parse the role of these two mechanistic hypotheses.

Although the primary molecular determinants of equilibrium selectivity are not yet resolved, chemists in the guest–host field and well-studied ion-binding proteins highlight an easy-to-calculate parameter that often correlates with the equilibrium preference for a cation: the size of the cage formed by the contacting oxygen atoms and the size of the dehydrated ion (Dietrich, 1985; Harding, 2002). This does not mean that the structure is rigid—as a rigid structure would not be able to enclose an ion—but that the energy minimum of the system is an oxygen-lined site that matches the size of the “site” created by the water molecules around a hydrated ion. Although this is a correlative parameter, not a mechanistic principle, a simple explanation is that the oxygen atoms in the ion-binding site mimic the hydration of water without the entropic penalty associated with ordering water molecules around an ion. Although the exact affinities vary depending on the channel and technique used to measure ion affinities, all examined K+ channels have equilibrium preference for K+ ions over Na+ ions, consistent with their preference to conduct K+ ions.

Nonselective tetrameric cation channels
Nonselective tetrameric cation channels including hyperpolarization-activated cyclic nucleotide–gated (HCN) and cyclic nucleotide–gated (CNG) channels show a low preference for K+/Na+ during ion conduction, as measured by reversal potential (Hille, 2001; Craven and Zagotta, 2006). While we await structures of bona fide members of these families, it is informative to compare the structural, transport, and equilibrium properties of the nonselective Bacillus cereus NaK and Vibrio parahaemolyticus TrkH channels with those of K+ channels. NaK is a homotetramer with an overall architecture similar to that of K+ and nonselective channels (Shi et al., 2006; Alam and Jiang, 2009a). From the channel’s cavity, ions enter oxygen-lined sites similar to sites S3 and S4 in K+ channels (Fig. 3 A). Upon leaving site S3, they encounter an expanded portion of the filter, with coordination by carbonyl oxygen atoms from site S3 and water molecules in what would be site S2 in K+ channels. An overlay of the crystal structures showed that the protein portion of the channel’s selectivity filter is the same in either Na+ or K+ ions. The ion-binding sites S3 and S4 in NaK are the same size as those found in the selectivity filter of K+ channels and K-selective small molecules, suggesting a transport and equilibrium-binding preference for K+ ions. However, a single-channel experiment demonstrates that the channel nonselectively conducts both K+ and Na+ ions, hinting that the transport properties of a channel cannot be gleaned from the size of the oxygen-lined cavities and ions in the selectivity filter (Derebe et al., 2011a). A free energy–perturbation MD study attributes the lack of transport selectivity of WT NaK channel to differences in hydration of bound ions in the pore compared with K+ channels (Noskov and Roux, 2007). The same study also revealed a slight equilibrium preference for K+ ions at site S3 and a slight Na+ preference at site S4. A different study points out that K+ ions tend to reside in the center of the oxygen cage, whereas Na+ ions prefer the plane of carbonyl oxygen atoms, which is also seen in KcsA K+ channels (Shen and Guo, 2009; Kim and Allen, 2011). An experimental determination of NaK channel selectivity at equilibrium has not been published to compare with these predictions.

Figure 3. The selectivity filter of the nonselective NaK and NaK2CNG-D channels. An Fo–Fc ion omit map (2σ) shows the electron density in the filter. (A) The WT NaK channel in the presence of K+ ions (PDB accession no. 3E8H). (B) Comparison of electron density in nonselective NaK2CNG-D channel. (Left) The selectivity filter of NaK2CNG-D with bound K+ ions (PDB accession no. 3K03). (Right) The selectivity filter of NaK2CNG-D with bound Na+ ions (PDB accession no. 3K04). Structures are from Alam and Jiang (2009b) and Derebe et al. (2011a).
NaK channels can be converted into CNG-like channels that are nonselective during ion conduction and are blocked by divalent cations by replacing their filter with the eukaryotic CNG channel’s selectivity filter sequence (Derebe et al., 2011a,b). Structures of these CNG-like channels (called NaK2CNG-X, where X is D, E, or N depending on the amino acid at position 66) show that both K’ and Na’ are able to bind within the selectivity filter (Fig. 3B). As with K’ channels, K’ ions prefer to bind in the oxygen cage, whereas Na’ ions are found in various positions throughout the filter. Potentials of mean force calculations showed that NaK2CNG-D is not selective for K’ or Na’ at equilibrium, potentially explaining the lack of transport selectivity (Wang et al., 2014). However, recent ITC and x-ray crystallography experiments revealed that the NaK2CNG-D and other mutant NaK channels have a large preference for K’ to Na’ ions at equilibrium (Liu and Lockless, 2013; Sauer et al., 2013).

Aside from the different number of oxygen-lined sites, CNG and K’ channels are remarkably similar but have different permeation preferences. Indeed, the nonselective NaK channel can be transformed into a K+-selective channel by swapping in the selectivity filter sequence of a K’ channel (TVGYGD), demonstrating that NaK protein is a reasonable system in which to study both K’ and nonselective ion conduction (Derebe et al., 2011a). This so-called NaK2K channel exhibits K+-selective ion conduction and a selectivity filter that is identical to the conductive conformations of all K’ channels determined to date. Not surprisingly, NaK2K also has an equilibrium preference for K’ ions, as demonstrated by x-ray crystallography and ITC (Liu and Lockless, 2013; Sauer et al., 2013). The primary structural difference between the nonselective CNG channel and the K’-selective NaK2K channel is that NaK2K has four sites, as is typical of K’ channels, whereas NaK2CNG-X channels have three sites.

The straightforward transformation of NaK channels to K’-like and CNG-like channels could partially explain pre-structure experiments in which deleting two amino acids from the Shaker K’ channels to mimic the sequence of CNG channels led to nonselective ion conduction and divalent block (Heginbotham et al., 1992). These CNG-converting mutations likely destroyed the mechanism whereby K’ channels translate their equilibrium preference into selective ion conduction (discussed in the next section), but, like NaK2CNG-D, select K’ ions at equilibrium. However, the number of observed sites in crystal structures does not always correlate with the permeation selectivity. For example, NaK2K(Y55F) has four binding sites but is nonselective during ion conduction (Sauer et al., 2011). A mutation in MthK to eliminate half of the oxygen atoms comprising site S4 renders the channel nonselective, but the equivalent mutation in Shaker retains the ability to selectively conduct K’ ions (Heginbotham et al., 1994; Derebe et al., 2011a). The lack of correlation between permeation selectivity and the number of binding sites in the queue suggests that additional parameters are needed to explain transport selectivity. Indeed, mutations of amino acids one shell away from the selectivity filter can alter the conduction, selectivity, and inactivation properties of channels with identical selectivity filter sequences, demonstrating that all information is not contained in the direct ion-binding ligands (Starkus et al., 1997; Cordero-Morales et al., 2006, 2011; Sauer et al., 2011; McCoy and Nimigean, 2012). In the future, it will be important to address the roles amino acids not directly contacting ions/substrates have in determining a transport protein’s selectivity.

Recent crystal structures of TrkH and KtrB suggest that the lack of correlation between selective ion transport and equilibrium binding is not an artifact of using engineered NaK mutant channels (Cao et al., 2011, 2013; Vieira-Pires et al., 2013). TrkH and its homologues were originally described as K’ transporters based on the observation that deleting them slowed or eliminated bacterial growth at low K’ concentrations (Dosch et al., 1991; Schlösser et al., 1995). Recent structural and functional data unambiguously show that TrkH is a nonselective channel (Cao et al., 2011, 2013). Unlike K’ channels and the NaK channel, the selectivity filter is formed from four nonidentical sequences that create ion-binding sites reminiscent of sites S3 and S4 in K’ channels and NaK channels; the resolutions of the available structures are too low to reliably determine the size of the binding sites, but the similar overall architecture to that of K’ channels and NaK channels suggests a K’ preference. The distantly related KtrB from Bacillus subtilis is remarkably similar in structure to TrkH, suggesting that KtrB is also a nonselective channel (Vieira-Pires et al., 2013). These data demonstrate that an equilibrium ion preference is not sufficient to explain ion transport selectivity in some channels, further calling into doubt our ability to predict transport properties from structures alone.

**Role for a queue of ions**

The conversion between K’-selective and nonselective channels by changing the number of binding sites suggests that the queue of ion-binding sites is critical for transport selectivity. Crystal structures of both K’-selective and nonselective tetrameric cation channels reveal queues of nearly identical ion-binding sites that prefer K’ ions to Na’ ions. The discrepancy between the selectivity of K’-selective and nonselective channels is at least partially resolved if we consider the queue of ion-binding sites in the selectivity filter instead of the properties of individual sites.

Several lines of evidence support the notion that the queue of K’-ion-binding sites is the relevant structural and functional unit in the K’ channel’s selectivity...
filter. First, the selectivity filter of KcsA undergoes ion-dependent conformational change; when K+ ions are replaced with Na+ ions, the filter rearranges to a constricted conformation that primarily retains sites S1 and S4 (Zhou et al., 2001). Second, the distributions of ions (K+, Rb+, Cs+, and Tl+) can vary from each other and can vary in different channels, suggesting a different energy profile along the whole selectivity filter, not simply each site individually (Morais-Cabral et al., 2001; Zhou and MacKinnon, 2003; Lam et al., 2014). Third, mutations that alter direct interactions with the ions can have different outcomes; a threonine-to-alanine mutation at site S4 of MthK K+ channel leads to loss of K+ selectivity, whereas the same mutation in Shaker K+ channel does not (Heginbotham et al., 1994; Derebe et al., 2011a).

Fourth, chemical modification of the backbone alters the distribution of Rb+ ions throughout the filter, not just near the modified site (Valiyaveetil et al., 2006; Matulef et al., 2013). Finally, the location of the channel blocker Ba2+ depends on whether Na+ or K+ is present and can differ in different channels (Lockless et al., 2007; Guo et al., 2014; Lam et al., 2014). Collectively, these data argue that the selectivity filter functions as a unit that binds a queue of ions and water, not as a series of independent sites.

Qualitative equilibrium properties of a queue can be inferred from studies of small, single-site K+-selective molecules. Structures of K+-selective cryptands, crown ethers, and antibiotics reveal that (a) ion-binding sites are similar in size to the ion’s hydration radius, and (b) they can deform to bind less preferred ion. These molecules bind K+ ions in a cage of oxygen atoms ~2.8 Å in diameter, but can deform to bind to the smaller Na+ ion with its optimal 2.4 Å diameter (Fig. 4) (Kilbourn et al., 1967; Dobler and Phizackerley, 1974; Neupertlavés and Dobler, 1975; Kauffmann et al., 1976; Steinrauf et al., 1982; Izatt et al., 1985; Cramp and Ho, 1986; Chekhlov, 2005a,c). Because many molecules change their shape depending on the bound ligand, it’s difficult to predict which ion would be optimally bound to a particular ion-binding site without knowing the equilibrium preference in advance. However, one might expect that if an ion-binding molecule does not change its shape in the presence of different ions, the size of the ion-binding site can be readily calculated from either structure. Additionally, if a structure is solved in different mixtures of ions, the most preferred ion would outcompete the less preferred ion to establish the size of the ion-binding molecule.

In the case of the K+-selective [2.2.2]cryptand, the distance between the Na+ ion and oxygen atoms averages 2.6 Å, which one would infer is not optimally bound (Chekhlov, 2005b) (Fig. 4). The non-optimal size of the ion-binding cavity may explain the lower affinity of the molecule for Na+ ions (Kauffmann et al., 1976). The inability of this [2.2.2]cryptand to deform to accommodate the smaller ion undoubtedly contributes to its high K+ selectivity. To adapt to the smaller Na+ ion, the oxygen atoms will either move in to reduce the size of the cage (causing strain), or the Na+ ion will need to bind in a different location than the center of the cage. The smaller Na+-selective [2.2.1]cryptand, with its slightly smaller cavity, prefers Na+ to K+ but is also able to bind both ions (Kauffmann et al., 1976; Cram and Ho, 1986). Thus, selectivity is the potential to bind an ion at the energy minimum, whose effects can be propagated to other parts of the structure or solvent. Either way, this disrupts the binding potential for a K+ ion that prefers to bind in the center of the cage with its own optimal coordination distance.

The selectivity filter is the equivalent of an enzyme-active site, so it is critical to consider its behavior as a connected unit in much the same way that an enzyme-active site is more than the sum of its parts. In both K+-selective and nonselective mutant NaK channels, Na+ and K+ ions bind to different sites across their selectivity filters (Derebe et al., 2011a,b). The occupancy of the sites is two to three K+ ions based on flux ratio measurements in several channels and in KcsA by comparing the electron density in K+ to the electron-dense thallium, whose occupancy is more easily obtained (Hodgkin and Keynes, 1955; Begenisich and De Weer, 1980; Spalding et al., 1981; Vestergaard-Bogind et al., 1985; Zhou and MacKinnon, 2003). Even with these constraints, there are a large number of possible models that could explain the selectivity and ion conduction of the channels. However, structures aid in eliminating possible models by highlighting a few reasonable assumptions. First, K+ ions bind in the center of the oxygen cage, in sites whose size is ideal for K+ ions. Second, Na+ ions can bind in the filter but do not necessarily occupy the same location as K+ ions (Zhou et al., 2001; Ye et al., 2010).

Figure 4. Crystal structures of K+-selective [2.2.2]cryptand. (Left) K+ ion is green with an average O–K+ distance of 2.8 Å. (Middle) Na+ ion is orange with an average O–Na+ distance of 2.6 Å. (Right) Li+ ion is yellow with an average O–Na+ distance of 2.4 Å. Structures are from Chekhlov (2003, 2005a,b).
This is likely because the selectivity filter does not provide optimal coordination along the entire length. Third, in mixtures of K⁺ and Na⁺, the electron density corresponding to K⁺ ions tends to be in the center of the oxygen cage (Zhou and MacKinnon, 2003; Ye et al., 2010; Sauer et al., 2013). This is consistent with the ITC observation that K⁺ has a higher affinity for the channels than Na⁺ and would indicate that the channel is “built” to bind queues of K⁺ ions over Na⁺ ions (Lockless et al., 2007; Liu et al., 2012; Liu and Lockless, 2013). Because K⁺ ions bind with higher affinity than Na⁺ ions, it is likely that K⁺ sets the “register” of the queue such that K⁺ ions will reside in the center of the oxygen cages at optimal coordination distances, whereas Na⁺ ions will be relegated to suboptimal positions.

In multi-ion queues, ions bound to selective sites influence one another’s conduction properties, which can lead to the well-known anomalous mole fraction effect (Hille and Schwarz, 1978; Almers and McCleskey, 1984; Hess and Tsien, 1984; Korn and Ikeda, 1995). In a K⁺ channel, the premise is that a K⁺ ion binds with high affinity when there is only one ion in the filter. The binding of a second ion, either Na⁺ or K⁺, will weaken the affinity of the queue of ions. If a Na⁺ ion is bound, the mixed ion queue encounters a barrier for the Na⁺ ion to move further into the filter, such that the queue is more likely to reset back to where it came from than allow the Na⁺ ion to transit further through the filter. The origin of the barrier is not clear, but several nonexclusive models have been proposed. A barrier could result from K⁺ ions residing longer in a site than Na⁺ ions, creating a physical barrier whereby the Na⁺ ion is unable to advance to an already occupied site (Hille and Schwarz, 1978; Korn and Ikeda, 1995). Intriguingly, a similar principle has been proposed to explain the nuclear pore complex’s selectivity for nuclear transport factor proteins (Zilman, 2009; Zilman et al., 2010). Or, the carbonyl oxygen atoms themselves could present a selective barrier to Na⁺ ion movement relative to K⁺ ions (Thompson et al., 2009). Alternatively, the location of the K⁺ ions in the center of the oxygen cage could force a Na⁺ ion to bind in a suboptimal position in its site, creating an environment where the ion is more likely to dissociate back than advance to the next site.

An extension of this idea is that destroying the multi-ion mechanism could lead to nonselective ion conduction. The ion selectivity and conduction properties of single- and multi-ion queues were widely discussed, long before structures were available (Bezanilla and Armstrong, 1972; Hille and Schwarz, 1978). In single-ion queues, the largest barrier encountered by each ion determines ion selectivity measured by relative conductances, whereas the rate of ion binding determines selectivity when measured by reversal potential. In essence, the ions move independently of one another, and their properties do not necessarily correlate with the equilibrium preferences of the channel. A discordance between the equilibrium and ion conduction selectivity was recently observed with mutant NaK channels that nonselectively conduct K⁺ or Na⁺ ions, yet retain their overall equilibrium selectivity for K⁺ ions (Liu and Lockless, 2013; Sauer et al., 2013). This suggests that these nonselective channels have a broken multi-ion mechanism, which might also explain how mutations in the KCNJ K⁺ channel and the GIRK2 channel and mutations to convert Shaker into a CNG-like channel resulted in nonselective conduction (Heginbotham et al., 1992; Slesinger et al., 1996; Choi et al., 2011). In each case, the equilibrium selectivity is likely preserved, but the transport selectivity is not. Perhaps the multi-ion mechanism is used to ensure equilibrium-based transport, as it is difficult to maintain selective barriers for similarly sized ions like Na⁺ and K⁺. An equilibrium-based transport mechanism also ensures that the desired ion is transported even if the local concentration of either ion changes significantly.

Multi-ion queues can also facilitate high ion conduction rates. Both K⁺ and nonselective channels are able to transport ions at rates approaching the diffusion limit (Hille, 2001). In other words, an ion is bound to the channel for ∼10 ns. This means that the channel must not bind the ions too tightly, as transport would be limited by the rate at which they can leave the channel. This is a particular problem for K⁺ channels, as the ion must reside in the channel long enough to discriminate between the similarly sized K⁺ and Na⁺ ions. The equilibrium ion-binding measurements in both K⁺ and nonselective channels reveal a K⁺ binding affinity in the mid-micromolar range, suggesting a $k_{off}$ rate of $10^5$ s⁻¹, which is too slow (Neyton and Miller, 1988; Baukrowitz and Yellen, 1996; Piasta et al., 2011; Liu et al., 2012; Liu and Lockless, 2013). Therefore, K⁺ ions must bind with lower affinity during ion conduction. One possible resolution to this conundrum is if the binding of multiple ions lowers the affinity of all ions in the queue, then selectivity is retained even at low affinities. The low millimolar-range affinities are outside of the experimental range for most equilibrium techniques but would be necessary to account for the high ion conduction rates.

A different problem exists with NaK2CNG-D channels, which are selective for K⁺ at equilibrium but nonselective during ion conduction. If K⁺ ions bind in the 10-µM range but Na⁺ ions bind in the 10-mM range, why do both ions go through the channel equally well in bi-ionic conditions (Derebe et al., 2011a)? One might expect that the K⁺ ion would block ion conduction because its high affinity would result in long residence times in the channel, unless its affinity is weakened from multiple ions interacting in the filter during ion conduction. This would then suggest that NaK2CNG-D is behaving similarly to K⁺ channels, but without selective ion conduction.
What then are the differences between NaK2CNG-D and K’ channels that lead to their divergent ion conductance selectivities?

Conclusion

The equilibrium ion-binding properties of ion channels and transporters can be difficult to discern from crystal structures alone, as proteins often adopt different lowest energy states depending on the ions bound. In cases where transport is slow, their inherent ion-binding preferences can be used to infer their transport preferences. However, in cases where transport is fast, the transport selectivity can hide their equilibrium preferences by accentuating the kinetics of ions hopping through a channel over its inherent ion-binding preferences. Thus, depending on the arrangement of ion-binding sites in a channel’s selectivity filter, one can achieve either selective or nonselective ion transport.

The equilibrium K’ selectivity of some nonselective channels suggests a potential mechanism whereby they could evolve into a fast K’-selective channel. K’ channels and nonselective channels like CNG and HCN are related to one another in both sequence and structure, suggesting an evolutionary link between them. Swap experiments show that only a few mutations separate a nonselective channel from a K’-selective channel. One might imagine an evolutionary path between these channels in which the equilibrium preference for a K’ ion in a nonselective channel evolves into a K’-selective channel through these few mutations to create the selective ion queue. Alternatively, a slow single-ion channel with an equilibrium and transport preference for K’ ions could be transformed into a fast multi-ion channel through mutations that create a queue of K’-selective ion-binding sites, as is seen in most K’ channels studied to date.

In the case of multi-ion selectivity filters, such as those found in K’ channels, the selectivity filter can be viewed as the active site that interacts with different queues of ions and water molecules. At least three properties emerge from multi-ion queues: (1) high conductance by reducing the affinity of multiple bound ions versus single ions; (2) high selectivity by allowing disfavored ions time to dissociate back into solution; and, consequently, (3) robust selectivity in an environment where ion concentrations can change. For transporters and carriers, the equilibrium preference and slow transport naturally create robust selectivity. In all these cases, equilibrium-based ion selectivity is achieved by slowing transport enough so that the disfavored ion is able to dissociate back into solution before transport takes place.

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REFERENCES

Alam, A., and Y. Jiang. 2009a. High-resolution structure of the open NaK channel. Nat. Struct. Mol. Biol. 16:30–34. http://dx.doi.org/10.1038/nsmb.1531
Alam, A., and Y. Jiang. 2009b. Structural analysis of ion selectivity in the NaK channel. Nat. Struct. Mol. Biol. 16:35–41. http://dx.doi.org/10.1038/nsmb.1537
Alam, A., and Y. Jiang. 2011. Structural studies of ion selectivity in tetrameric cation channels. J. Gen. Physiol. 137:397–403. http://dx.doi.org/10.1085/jgp.201010546
Almers, W., and E.W. McCleskey. 1984. Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. J. Physiol. 353:585–608. http://dx.doi.org/10.1113/jphysiol.1984.sp01552
Baconguis, I., and E. Gouaux. 2012. Structural plasticity and dynamic selectivity of acid-sensing ion channel-spider toxin complexes. Nature. 489:400–405. http://dx.doi.org/10.1038/nature11375
Bakewitz, T., and G. Yellen. 1996. Use-dependent blockers and exit rate of the last ion from the multi-ion pore of a K’ channel. Science. 271:653–656. http://dx.doi.org/10.1126/science.271.5249.653
Begenisich, T., and P. De Weer. 1980. Potassium flux ratio in voltage-clamped squid giant axons. J. Gen. Physiol. 76:83–98. http://dx.doi.org/10.1085/jgp.76.1.83
Benezilla, F., and C.M. Armstrong. 1972. Negative conductance caused by entry of sodium and cesium ions into the potassium channels of squid axons. J. Gen. Physiol. 60:588–608. http://dx.doi.org/10.1085/jgp.60.5.588
Bhate, M.P., B.J. Wylie, L. Tian, and A.E. McDermott. 2010. Conformational dynamics in the selectivity filter of KcA in response to potassium ion concentration. J. Mol. Biol. 401:155–166. http://dx.doi.org/10.1016/j.jmb.2010.06.031
Blatz, A.L., and K.L. Magleby. 1984. Ion conductance and selectivity of single calcium-activated potassium channels in cultured rat muscle. J. Gen. Physiol. 84:1–23. http://dx.doi.org/10.1085/jgp.84.1.1
Boudker, O., R.M. Ryan, D. Yernool, K. Shimamoto, and E. Gouaux. 2007. Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. Nature. 445:387–393. http://dx.doi.org/10.1038/nature05455
Brohawn, S.G., J. del Marmol, and R. MacKinnon. 2012. Crystal structure of the human K2P TRAAK, a lipid- and mecano-sensitive K’ ion channel. Science. 335:436–441. http://dx.doi.org/10.1126/science.1213808
Cao, Y., X. Jin, H. Huang, M.G. Derebe, E.J. Levin, V. Kabaleeswaran, Y. Pan, M. Punta, J. Love, J. Weng, et al. 2011. Crystal structure of a potassium ion transporter, TrkH. Nature. 471:336–340. http://dx.doi.org/10.1038/nature09731
Cao, Y., Y. Pan, H. Huang, X. Jin, E.J. Levin, B. Kloss, and M. Zhou. 2013. Gating of the TrkH ion channel by its associated RCK protein TrkA. Nature. 496:317–322. http://dx.doi.org/10.1038/nature12056
Chekhlov, A.N. 2003. Crystal structure of (2,2.2-cryptand)lithium perchlorate. Russ. J. Coord. Chem. 29:828–832. http://dx.doi.org/10.1023/B:RUCO.0000083933.57920.7d
Chekhlov, A.N. 2005a. Synthesis and crystal structure of (2,2.2-cryptand)potassium bicarbonate trihydrate. Russ. J. Inorg. Chem. 50:1556–1560.
Lam, Y.L., W. Zeng, D.B. Sauer, and Y. Jiang. 2014. The conserved

Korn, S.J., and S.R. Ikeda. 1995. Permeation selectivity by compe-

Liu, S., and S.W. Lockless. 2013. Equilibrium selectivity alone
does not create K+-selective ion conduction in K+ channels. Nat.

Liu, S., X. Bian, and S.W. Lockless. 2012. Preferential binding of K+-ions in the selectivity filter at equilibrium explains high selec-
tivity of K+ channels. J. Gen. Physiol. 140:671–679. http://dx.doi.
or/10.1085/jgp.201210855

Lockless, S.W., M. Zhou, and R. MacKinnon. 2007. Structural and thermodynamic properties of selective ion binding in a K+ channel. PLoS Biol. 5:e121. http://dx.doi.org/10.1371/journal.
.phio.0050121

Long, S.B., E.B. Campbell, and R. MacKinnon. 2005. Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science. 309:897–903. http://dx.doi.org/10.1126/science.1116290

Long, S.B., X. Tao, E.B. Campbell, and R. MacKinnon. 2007. Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. Nature. 450:376–382. http://dx.doi.
or/10.1038/nature06265

Luzhkov, V.B., and J. Apvist. 2001. K+ /Na+ selectivity of the KcsA potassium channel from microscopic free energy perturbation calculations. Biochim. Biophys. Acta. 1548:194–202. http://dx.doi.
or/10.1016/S0167-4888(01)00213-8

Mari, S.A., J. Pessoa, S. Altieri, U. Hensen, L. Thomas, J.H. Morais-
Cabral, and D.J. Müller. 2011. Gating of the MioI/KI potassium channel involves large rearrangements of the cyclic nucleotide-

Matoúf, K., A.G. Komarov, C.A. Costantino, and F.I. Valivette.
2013. Using protein backbone mutagenesis to dissect the link between ion occupancy and β-type inactivation in K+ channels. Proc. Natl. Acad. Sci. USA. 108:20802–20807. http://dx.doi.org/10.1073/pnas.111149108

Matcovy, J.G., and C.M. Noskov. 2012. Structural correlates of se-

Miller, A.N., and S.B. Long. 2012. Crystal structure of the human
two-pore domain potassium channel K2P1. Science. 335:432–436. http://dx.doi.org/10.1126/science.1213274

Moore, C., and B.C. Pressman. 1964. Mechanism of action of valino-
mycin on mitochondria. Biochem. Biophys. Res. Commun. 15:562– 567. http://dx.doi.org/10.1016/0006-291X(64)90505-4

Morais-Cabral, J.H., Y. Zhou, and R. MacKinnon. 2001. Energetic optimization of ion conduction rate by the K+ selectivity filter. Nature. 414:37–42. http://dx.doi.org/10.1038/35102000

Neupert-Laves, K., and M. Dobler. 1975. The crystal structure of a K+ complex of valinomycin. Helv. Chim. Acta. 58:432–442. http://dx.doi.org/10.1002/hlca.19750580212

Neyton, J., and C. Miller. 1988. Discrete Ba2+ block as a probe of ion occupancy and pore structure in the high-conductance Ca2+- activated K+ channel. J. Gen. Physiol. 92:569–586. http://dx.doi.org/10.1085/jgp.92.5.569

Noskov, S.Y., and B. Roux. 2006. Ion selectivity in potassium chan-
nels. Biophys. Chem. 124:279–291. http://dx.doi.org/10.1016/j.bipec.
.2006.05.033

Noskov, S.Y., and B. Roux. 2007. Importance of hydration and dyn-
amics on the selectivity of the KcsA and NaK channels. J. Gen.
Physiol. 129:135–143. http://dx.doi.org/10.1085/jgp.200609633

Noskov, S.Y., S. Bernèche, and B. Roux. 2004. Control of ion select-

Nyblom, M., H. Poulsen, P. Gourdon, L. Andersson, E. Lindahl, N. Fedosova, and P. Nissen. 2013. Crystal structure of Na+, K+-ATPase in the Na+-bound state. Science. 342:125–127. http://dx.doi.org/10.1126/science.1243552

Piasta, K.N., D.L. Theobald, and C. Miller. 2011. Potassium-selective block of barium permeation through single KcsA channels. J. Gen. Physiol. 138:421–436. http://dx.doi.org/10.1085/jgp.
.201110684

Picollo, A., M. Malvezzi, J.C. Houtman, and A. Accardi. 2009. Basis of substrate binding and conservation of selectivity in the CLC family of channels and transporters. Nat. Struct. Mol. Biol. 16:1294–1301. http://dx.doi.org/10.1038/nsmb.1704

Ressl, S., A.C. Tervisch van Scheltinga, C. Vonrhein, V. Ott, and C. Ziegler. 2009. Molecular basis of transport and regulation in the Na+/betaine symporter BetP. Nature. 458:47–52. http://dx.doi.org/10.1038/nature07819

Reyes, N., S. Oh, and O. Boudker. 2013. Binding thermodynamics of a glutamate transporter homolog. Nat. Struct. Mol. Biol. 20:634– 640. http://dx.doi.org/10.1038/nsmb.2548

Roux, B., S. Bernèche, B. Eggwolf, B. Lev, S.Y. Noskov, C.N. Rowley, and H. Yu. 2011. Perspectives on: Ion selectivity: Ion selectivity in channels and transporters. J. Gen. Physiol. 137:415–426. http://dx.doi.org/10.1085/jgp.201010577

Sauer, D.B., W. Zeng, S. Raghunathan, and Y. Jiang. 2011. Protein interactions central to stabilizing the K+ channel selectivity filter in a four-sited configuration for selective K+ permeation. Proc. Natl. Acad. Sci. USA. 108:16634–16639. http://dx.doi.org/10.1073/pnas.
.1111688108

Sauer, D.B., W. Zeng, J. Canty, Y. Lam, and Y. Jiang. 2013. Sodium and potassium competition in potassium-selective and non-selec-
tive channels. Nat. Commun. 4:3721. http://dx.doi.org/10.1038/ncomms3721

Schlösser, A., M. Meldorf, S. Stumpf, E.P. Bakker, and W. Epstein. 1995. TrkH and its homolog, TrkG, determine the specificity and kinetics of cation transport by the Trk system of Escherichia coli. J. Bacteriol. 177:1908–1910.
Shen, R., and W. Guo. 2009. Ion binding properties and structure stability of the NaK channel. *Biochim. Biophys. Acta.* 1788:1024–1032. http://dx.doi.org/10.1016/j.bbamem.2009.01.008

Shi, N., S. Ye, A. Alam, L. Chen, and Y. Jiang. 2006. Atomic structure of a Na\(^+\)- and K\(^+\)-conducting channel. *Nature* 440:570–574. http://dx.doi.org/10.1038/nature04508

Slesinger, P.A., N. Patil, Y.J. Liao, Y.N. Jan, L.Y. Jan, and D.R. Cox. 1996. Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K\(^+\) channels. *Neuron* 16:321–331. http://dx.doi.org/10.1016/S0896-6273(00)80050-1

Spalding, B.C., O. Senyk, J.G. Swift, and P. Horowitz. 1981. Unidirectional flux ratio for potassium ions in depolarized frog skeletal muscle. *Am. J. Physiol.* 241:C68–C75

Starkus, J.G., L. Kuschel, M.D. Rayner, and S.H. Heinemann. 1996. Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K\(^+\) channels. *Neuron* 16:321–331. http://dx.doi.org/10.1016/S0896-6273(00)80050-1

Thompson, A.N., I. Kim, T.D. Panosian, T.M. Iverson, T.W. Allen, Shi, N., S. Ye, A. Alam, L. Chen, and Y. Jiang. 2006. Atomic structures to enable selective partitioning. *Biophys. J.* 90:1085–1091. http://dx.doi.org/10.1016/j.bpj.2005.10.008

Steinrauf, L.K., J.A. Hamilton, and M.N. Sabesan. 1982. Crystal structure of valinomycin-sodium picrate. Anion effects on valinomycin-cation complexes. *J. Am. Chem. Soc.* 104:4085–4091. http://dx.doi.org/10.1021/ja00379a008

Tao, X., J.L. Avalos, J. Chen, and R. MacKinnon. 2009. Crystal structure of a bacterial homologue of Na\(^+\)/Cl\(^-\)-dependent symporter-1 family transporter. *Science* 327:709–713. http://dx.doi.org/10.1126/science.1164440

Whorton, M.R., and R. MacKinnon. 2011. Crystal structure of the mammalian GIRK2 K\(^+\) channel and gating regulation by G proteins, PIP\(_2\), and sodium. *Cell* 147:199–208. http://dx.doi.org/10.1016/j.cell.2011.07.046

Weyand, S., T. Shimamura, S. Yajima, S. Suzuki, O. Mirza, K. Krasous, E.P. Carpenter, N.G. Rutherford, J.M. Hadden, J. O’Reilly, et al. 2008. Structure and molecular mechanism of a nucleobase-cation-symport-1 family transporter. *Science* 322:709–713. http://dx.doi.org/10.1126/science.1164440

Wang, Y., A.C. Chamberlin, and S.Y. Noskov. 2014. Molecular strategies to achieve selective conductance in NaK channel variants. *J. Phys. Chem. B.* 118:2041–2049.

Ye, S., Y. Li, and Y. Jiang. 2010. Novel insights into K\(^+\) selectivity from high-resolution structures of an open K\(^+\) channel pore. *Nat. Struct. Mol. Biol.* 17:1019–1023. http://dx.doi.org/10.1038/nsmb.1865

Zhou, X., E.J. Levin, Y. Pan, J.G. McCoy, R. Sharma, B. Kloss, R. Bruni, M. Quick, and M. Zhou. 2014. Structural basis of the alternating-access mechanism in a bile acid transporter. *Nature* 505:569–573. http://dx.doi.org/10.1038/nature12811

Zhou, Y., and R. MacKinnon. 2005. The occupancy of ions in the K\(^+\) selectivity filter: Charge balance and coupling of ion binding to a protein conformational change underlie high conduction rates. *J. Mol. Biol.* 353:963–975. http://dx.doi.org/10.1016/j.jmb.2005.09.022

Zhou, Y., and R. MacKinnon. 2004. Ion binding affinity in the cavity of the KcsA potassium channel. *Biochemistry* 43:4978–4982. http://dx.doi.org/10.1021/bi049876z

Zhou, Y., J.H. Morais-Cabral, A. Kaufman, and R. MacKinnon. 2001. Chemistry of ion coordination and hydration revealed by a K\(^+\) channel-Fab complex at 2.0 Å resolution. *Nature* 414:43–48. http://dx.doi.org/10.1038/35102009

Zilman, A. 2009. Effects of multiple occupancy and interparticle interactions on selective transport through narrow channels: Theory versus experiment. *Biophys. J.* 96:1235–1248. http://dx.doi.org/10.1016/j.bpj.2008.09.058

Zilman, A., S. Di Talia, T. Jovanovic-Talisman, B.T. Chait, M.P. Rout, and M.O. Magnasco. 2010. Enhancement of transport selectivity through nano-channels by non-specific competition. *PLOS Comput. Biol.* 6:e1000804. http://dx.doi.org/10.1371/journal.pcbi.1000804

Zimmermann, I., and R. Dutzler. 2011. Ligand activation of the prokaryotic pentameric ligand-gated ion channel ELIC. *PLoS Biol.* 9:e1001101. http://dx.doi.org/10.1371/journal.pbio.1001101