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Ion selectivity in potassium channels

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Abstract

Potassium channels are tetrameric membrane-spanning proteins that provide a selective pore for the conduction of K^+ across the cell membranes. One of the main physiological functions of potassium channels is efficient and very selective transport of K^+ ions through the membrane to the cell. Classical views of ion selectivity are summarized within a historical perspective, and contrasted with the molecular dynamics (MD) simulations free energy perturbation (FEP) performed on the basis of the crystallographic structure of the KcsA phospholipid membrane. The results show that the KcsA channel does not select for K^+ ions by providing a binding site of an appropriate (fixed) cavity size. Rather, selectivity for K^+ arises directly from the intrinsic local physical properties of the ligands coordinating the cation in the binding site, and is a robust feature of a pore symmetrically lined by backbone carbonyl groups. Further analysis reveals that it is the interplay between the attractive ion–ligand (favoring smaller cation) and repulsive ligand–ligand interactions (favoring larger cations) that is the basic element governing Na^+/K^+ selectivity in flexible protein binding sites. Because the number and the type of ligands coordinating an ion directly modulate such local interactions, this provides a potent molecular mechanism to achieve and maintain a high selectivity in protein binding sites despite a significant conformational flexibility.

Keywords: Molecular dynamics; Potassium; Sodium; Solvation; Hydration; Membrane; Proteins

1. Introduction

Potassium channels are membrane-spanning proteins that provide an energetically favorable pathway for the selective conduction of K^+ ions across the membrane [1]. One of the most striking properties of potassium channels is their remarkable ability to conduct K^+ ions near the diffusion limit and yet, select for K^+ over Na^+ by more than ~1000 to 1. Because small ions such as Na^+ and K^+ are strongly bound to water molecules in bulk solution, the channel provides coordinating groups that help compensate the loss of hydration. Selectivity arises when this energetic compensation is more favorable for one type of ion than for another, relative to the hydration free energy. The most relevant ions in biological systems are Na^+, K^+, Cl^-, and Ca^{2+}, but Na^+ and K^+ are the most abundant, with a high intracellular concentration for K^+ and a high extracellular concentration for Na^+. The molecular mechanism underlying the rapid discrimination between K^+ and Na^+ is, therefore, fascinating because these two monovalent cations are very similar, differing only slightly in their atomic radius (by ~0.38 Å) [2].

The determination of the three-dimensional structure of K^+ channels at atomic resolution using X-ray crystallography [3–5] provides a unique opportunity to deepen our understanding of these systems. The X-ray structure of the KcsA bacterial K^+ channel from *Streptomyces lividan*, shown in Fig. 1, revealed that the narrowest region of the pore is lined by backbone carbonyl groups from the residues of "signature" sequence TTVGYG common to all known potassium channels [3]. In the narrow selectivity filter, K^+ must be almost completely dehydrated. These observations led to a commonly accepted explanation of ion selectivity, which assumes that structural factors play the dominant role [3,6–12]. It is interesting to see how the common view goes back to ideas expressed by Mullins, [13] and Bezanilla and

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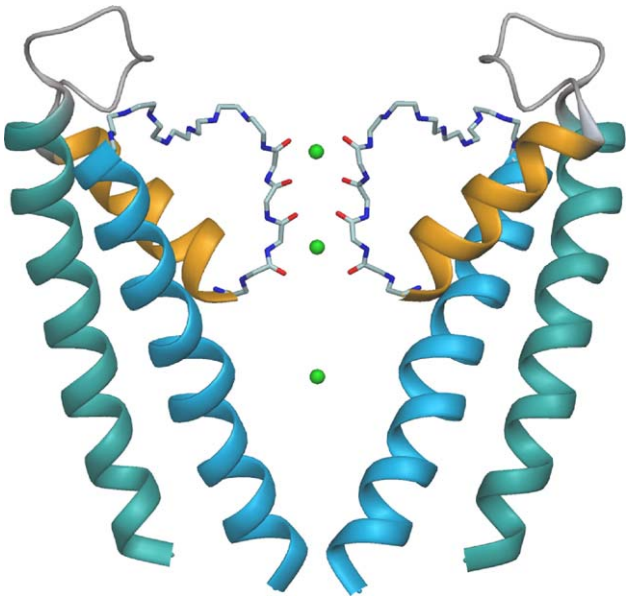

Fig. 1. Schematic structure of K^+ channel (KcsA) with 3 ions in cavity, S3 and S1 sites of the selectivity filter (shown in sticks).

Armstrong [14] several decades ago. It is also interesting to review alternative explanations that were proposed [15] and considered seriously [16].

Questions about ion selectivity have fascinated researchers for decades. Many investigators, with many different ideas, have contributed to frame the current view of ion selectivity [13,14,16,17]. More recently, the development of computational approaches based on sophisticated all-atom molecular dynamics simulations [18–23] has started to offer a "virtual route" for testing various ideas about the molecular mechanism of ion selectivity. One of our goals with this review, in addition to review the recent results from modern computations, will be to provide a historical context in which the various contributions were introduced and debated. Because the remarkable insight displayed by many of the previous authors can only be fully appreciated by reading what they wrote in their own words, we make a special effort to cite them as literally as possible.

2. Basic experiments and observations

In the simplest terms, selectivity reflects the fact that an "undesired" ion encounters more difficulty than a "desired" ion when it attempts to go through the channel, i.e., it experiences an environment that is energetically unfavorable (relative to the bulk). In this sense, ion selectivity is first and foremost about energy. But selectivity may manifest itself in different ways, depending on whether it is experimentally probed using equilibrium binding measurements, or non-equilibrium flux and ionic current measurements [1,24]. Some types of measurements are more sensitive to the free energy at the bottom of a binding site, whereas other types of experiment are more sensitive to the height of free energy barriers. Classically, the selectivity of ion channels has been characterized from the reversal potential (zero net current) under bionic conditions. According to the Goldman–Hodgkin–Katz equation (GHK) [1],

$$V_{out} - V_{in} = \frac{k_B T}{q} \ln\left[\frac{P_K[K]_{in} + P_{Na}[Na]_{in} + P_{Cl}[Cl]_{out}}{P_K[K]_{out} + P_{Na}[Na]_{out} P_{Cl}[Cl]_{in}}\right] \quad (1)$$

where P_i are the permeability coefficients of the various ionic species. Assuming that a cationic channel is impermeable to anions (P_{Cl} is zero), the shift in reversal potential relative to the Nernst potential is thus dominated by the permeability ratio P_{Na}/P_K. In principle, the permeability ratio of Na^+ to K^+ can be measured from the reversal potential. However, this method can be difficult to use if the ratio P_{Na}/P_K is extremely small. In this case, electrophysiological measurements with blockade relief become more effective to quantitatively characterize the selectivity of K^+ channels. Ba^{2+} blockade experiments were used by Neyton and Miller [25,26] to detect and characterize the ion binding sites in the pore of K^+ channels. From the K^+ and Na^+-concentration dependence of the Ba^{2+} blockades, they discovered a K^+ binding site in the selectivity filter, called "external lock-in site" and estimated its free energy relative to Na^+ to be around +5.5 kcal/mol. Theoretical studies of ion conduction through the KcsA channel, in accord with the results of Neyton and Miller, suggest that the most plausible location of this binding site is either the S1 or S2 binding sites [22,23,28]. Recent quantum-chemical computations at the Hartree-Fock level also identify the binding site S_2 as the most stable location [29]. An alternative approach to provide quantitative information about the relative free energy of different cations in the selectivity filter is to perform "punchthrough" experiments with Na^+ on the intracellular side. In punchthrough experiments, the intracellular Na^+ gives rise to an apparent voltage-dependent block, which becomes relieved at (inside positive) high voltage as the escape of the blocker through the selectivity filter is accelerated. The punchthrough experiments yield typical "S"-shaped IV curves displaying a minimum in the current at some voltage, beyond which it starts to increase

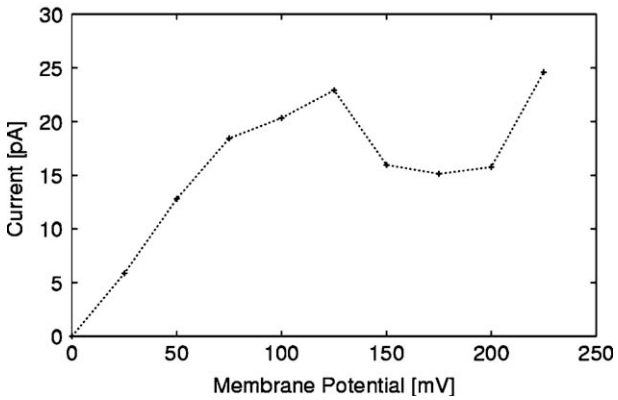

Fig. 2. Results from BD simulations with 200 mM intracellular Na^+ and 250 mM symmetric K^+ based on the PMF calculated for K^+ [22,28]. It was assumed that the free energy profile for Na^+ relative to K^+ reaches a maximum of about 5–6 kcal/mol in the site S_2 at the center of the selectivity filter, decreasing to zero at the intracellular and extracellular ends of the pore, in the sites S_4 and S_0.

again. Punchthrough experiments performed on wild-type KcsA by Nimigean and Miller with 200 mM Na^+ and symmetric 250 mM K^+ display this feature [27]. They observed that the voltage-dependent block by intracellular Na^+ increased up to about 200 mV and was relieved at a higher voltage. To illustrate the phenomenon, we generated Brownian Dynamics (BD) trajectories under the same conditions using the multi-ion PMF calculated from all-atom MD [22,28]. The results of the punchthrough-BD are illustrated in Fig. 2. The main qualitative features of the experiments, with S-shape IV curve and relief of the internal Na^+ blockade at high voltages applied to the membrane around ~200 mV [30], can be reproduced by assuming that free energy profile of Na^+ is slightly more unfavorable than that of K^+, with $\Delta\Delta G$ reaching a maximum of ~6 kcal/mol at the binding site S_2 and decreasing at the intracellular and extracellular ends of the selectivity filter.

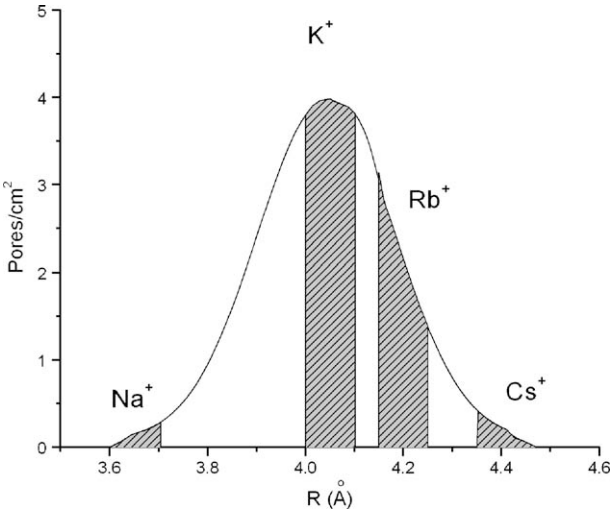

Fig. 3. Fitted distribution of the pore number (monotonic, i.e. same sized pores in the assumption of L.J. Mullins) as a function of given ion radius. Ion radius was determined as following = crystal radius + 2.72 Å (size of 1 water molecule).

3. Historical perspective

Early studies of ion selectivity in the 1930s were focused on inorganic systems such as aluminosilicates, minerals or synthetic resins. Observed selectivity patterns, or "selectivity sequences", were explained on the basis of differences in ionic Pauling radii or Stokes–Einstein hydrodynamic radii [31]. Despite its simplicity and overall attractiveness, additional studies of monovalent ion selectivity in different inorganic systems revealed that many other patterns co-existed in addition to already known ones [32]. Indeed, these observations could not be accounted for within the framework of Jenny's hypothesis [29] because the selectivity sequences in some systems (aluminosilicates, resins and certain zeolites) were not directly related to the either the Pauling ionic radii or the hydrated ionic radii [32]. Gregor [33] attempted to revisit the theory of Jenny [31] to explain monovalent cation selectivity in a series of synthetic resins. They proposed that selectivity for two ions in chemical equilibrium arises as a result of differences in hydration volumes and energies required to strip water while the ion remained inside the exchanger. The major problem of this explanation was the difficulty in explaining differences in selectivity between ions of similar radii and with closely related hydrodynamic properties (for example K^+ versus Na^+) without having to arbitrarily adjust the size of hydrated ions.

3.1. Narrow cylindrical pores

One of the first attempts to provide a structural explanation of the mechanism of selectivity in biological systems was made by Mullins [13]. Based on electrophysiological measurements of monovalent cation influx into *frog sartorius* muscle (Na^+, K^+, Rb^+ and Cs^+), he noted a relationship between membrane conductance and ion size (Fig. 3). Mullins was fully aware of the large magnitude of hydration energies, stating that "*the hydration energies of Na^+ and K^+ are about 95 and 75 kcal/mol, and as these energies are of the order of the strength of stable chemical bonds, it seems necessary to conclude that the observed influxes of these ions cannot be due to the escape of the ions from the hydration*". He also realized the constraints hydration puts on ion permeation: "*If an ion is to penetrate through a membrane composed of small pores, it must replace the water molecules that are serving as hydration with other molecules (the pore walls)…*". To explain the observed differences in ion selectivity, Mullins postulated the existence of "*cylindrical pores*" spanning the membrane. Assuming that ions surrounded by 0, 1 or 2 complete shells of water molecules would maintain "*monotonic*" and "*circular profiles*", he argued that such a cylindrical channel might select a partially hydrated ion of an optimal size while discriminating over smaller or larger ions: "*If K^+ approaches a pore that is precisely the same size as this ion with its first solvation shell, it may, as indicated previously, exchange hydration, for water shells from 2 to infinity, for a similar attraction with the structure lining the pore. If the pore is somewhat smaller that K^+ penetration cannot occur for steric reasons, while if pore is somewhat too large, penetration likewise cannot occur because the attraction of the ion for water shells of 2 and greater is not compensated by solvation of similar magnitude in the pore*" [13,34,35].

The ideas of Mullins had long-lasting impact. In particular, the concept of partially hydrated ions being discriminated by a narrow pore of a given radius remains broadly valid and has been a cornerstone of theories of selective ion permeation ever since. Nonetheless, several aspects were problematic. For example, Diamond and Wright [36] pointed out that the concept of "*similar attraction*", i.e., the assumption that any neighboring atom at a given distance must give rise to similar interactions and forces, was unphysical and unjustified. Clearly, the concept served one purpose in the arguments of Mullins: to avoid the complexity of microscopic interactions and enable an explanation of ion size-dependent selectivity on the sole basis of geometrical considerations. The simple concept of geometrical "misfit" of a smaller cation such as Na^+ ion in a nearly rigid binding site optimally designed to bind the larger K^+ ion has been frequently invoked to explain selectivity in host/guest chemistry and cyclic antibiotics as a function of ion radius and

antibiotic ring sizes [37,38]. Though experimental studies of different macrocyclic compounds showed no simple relation between ring size and ion selectivity patterns [39,40], such ideas remain very popular to this day.

3.2. Field strength model of ion selectivity

In the early 1960s, Eisenman [17] proposed a mechanism for equilibrium selectivity of glass electrodes. Eisenman considered the electrostatic energy difference ΔE caused by the exchange of one water molecule (dehydration) for one ligand coordinating an ion:

$$\Delta E = E_{\text{ion-ligand}} - E_{\text{ion-water}} \quad (2)$$

where $E_{\text{ion-ligand}}$ and $E_{\text{ion-water}}$ are the interactions of between the ion–ligand and ion–water interactions, respectively. The energies were estimated using experimental dipole strengths for the ligand and water molecule assuming interatomic distances from Pauling crystal radii. In this simple model, the selectivity of a binding site arises from the relative differences in energy ΔE for different cations, multiplied by the total number of exchanged ligands. The selectivity for Li^+, Na^+, K^+, Rb^+, and Cs^+ for a carbonyl-like ligand is illustrated in Fig. 4. The trends can be reversed from the low to the field limit depending on the dipole assigned to the ligand.

Based on such remarkably simple considerations, Eisenman was able to predict all the experimentally observed selectivity sequences of glass electrodes with simple variation of the dipole moment (or partial charges) of the ligands. He also pointed out that small variations in energy would arise naturally and that these variations would not be a monotonic function of the ion radius because of differences in local chemical structure between ligands and water molecules. Eisenman highlighted the importance of "field strength" as a determining factor in ion size selectivity, "*Increasing the field strength (which corresponds to an increase in dipole moment) yields a decrease in selectivity for larger ions relative to K^+….In contrast, the restrains in packing about the ion due to the increase bulkiness of the solvent molecules would be expected to discriminate more against the smaller ions…*". If the field strength is greater than the corresponding hydration energy, then the cation with the smallest (dehydrated) radius will be selected. In contrast, a low-field site will be selective for ions with larger ionic radii.

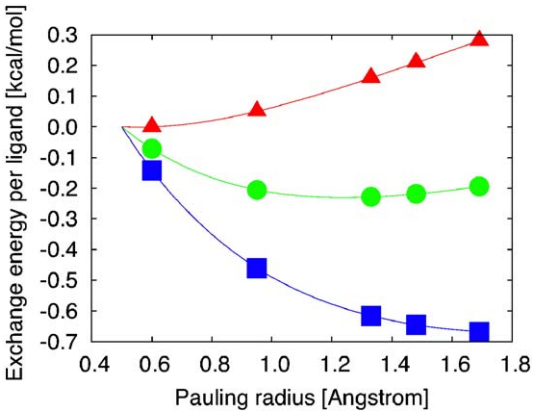

Fig. 4. Energy difference caused by removing one water molecule in the first hydration shell of a monovalent cation and replacing it by a carbonyl-like group. The energy is shown for different electric dipoles for the purpose of illustrating the effect of high (red triangles), medium (green circles) and low (blue squares) field strengths on ion selectivity. A dipole of 1.85 D was assumed for the water molecule and dipoles of 2.45, 2.39 and 2.33 D were used to represent the high, medium and low fields, respectively. The circles represent Li^+, Na^+, K^+, Rb^+, and Cs^+ using classical Pauling radii for the cations [2]. The curves were shifted to yield a difference of zero at a radius of 0.5 Å for the sake of clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The initial ideas about field strength were developed on the basis of a fixed binding site, but later, Eisenman and Krasne pointed out that molecular structures are dynamic [15]. Assuming that the local environment corresponding to ion binding sites in the interior of ionophore molecules were somewhat similar to the solvation by an effective solvent, Eisenman and Krasne [15] examined various organic solvents with oxygen atoms of different dipole strength (hydroxyl, ether, amide, ester, etc.). Collating equilibrium thermochemical heats of transfer of ions between water and these solvents, Eisenman and Krasne concluded that K^+ selective sites might comprise amide carbonyl from the backbone of proteins. The idea that any useful information about the mechanism of selectivity of a binding site in a biological molecule could be gleamed from the thermodynamics of ion solvation in a liquid, i.e., a dynamical environment that does not impose any specific geometric structure of the ligand coordinating the ion, has important implications. The intrinsic properties of the coordinating ligands have an important impact on size selectivity, regardless of the architectural rigidity of the binding site. Nonetheless, the relative importance of architectural rigidity of the binding site on size selectivity remained an unresolved issue, even in the later work by Eisenman and Alvarez [41].

3.3. Snug-fit mechanism of ion selectivity in K^+ channels

A structural explanation somewhat related to the model of Mullins was proposed in 1972 by Bezanilla and Armstrong [14]. Bezanilla and Armstrong described K^+ channels as pores with the wide and non-selective vestibule capable of accommodating a variety of monovalent cations, tetraethylammonium ion (TEA^+) and other ions. K^+ ion in the vestibule or cavity remains fully hydrated and the pore discriminates against other ions in the narrowest section next to vestibule with a diameter of 2.6 to 3.0 Å. The authors suggested that [14]: "*The oxygens are fixed rigidly in positions that provide a good fit for a K^+ ion.… The distance from Na^+ to two of the oxygens of the cage is greater than would be the case for Na^+ in water, and the coulombic energy of an Na^+ ion in the cage is thus much higher than in water. Because of its relatively high energy in the cage, Na^+ would be unlikely to enter…*". Another important assumption regarding the energetics and average ion–ligand distances in the bulk phase and pore environment was also made "*…Because the center-to-center distance from oxygen to K^+ in water and in the cage is the same, that portion of coulombic energy of the K^+ ion which depends on interaction is the same*

in both situation" [14]. A simple graphic representation of their model is plotted in Fig. 5.

3.4. "Snug-fit" versus "field strength"

The most important concept from Bezanilla and Armstrong [14] is that a K^+ ion must be almost completely dehydrated within the structural confinement of a narrow pore in order to be "*recognized*" by the protein. This is in contrast with Mullins [13], who assumed that permeating ions had to be partially hydrated. The most important contribution from Eisenman regards energetic and thermodynamic factors rather than structural ones [15]. As his work made clear [15,17], the assumption of Mullins [13] that any neighboring atom at a given distance must give rise to similar interactions and forces ("*similar attraction*") is incorrect: the oxygens of water molecules surrounding a cation in the solvent are not electrostatically equivalent to the oxygens (from either hydroxyls or carbonyls groups) coordinating a cation in a binding site. The key concept is the "field strength" of the oxygen ligand. Eisenman was the first to test his ideas with calculations based on simple atomic models, and this is perhaps the most important legacy of his work.

In retrospect, it is clear that both the "snug-fit" mechanism of Bezanilla and Armstrong [14] and the "field strength" model of Eisenman and Krasne [15] captured some essential aspects of selectivity in ion channels. Nonetheless, at the time they appeared to present opposing views about selectivity. For example, Bezanilla and Armstrong [14] argued that the concept of field strength would be applicable only for case of selective ion equilibrium binding as in the case of ionophore carrier molecules, but not for non-equilibrium situation such as permeation through an ion channel. They constructed a simple one-site two-barrier pore to show that variations in the equilibrium selectivity of the central site need not affect ion flux selectivity. While their model illustrated their point about the binding site, it allowed only for a change of free energy at the bottom of the wells. It did not consider that the concept of field strength could (and should) also be applied to the top of a free energy barrier. Eisenman and Horn [42] were able to show that inclusion of a non-equilibrium diffusion component into the model did not affect the main conclusions about balances between dehydration and interaction in the ion binding sites as major source of K^+/Na^+ selectivity.

In an insightful discussion, Hille [16] compared and contrasted the "snug-fit" mechanism of Bezanilla and Armstrong [14] and the "field strength" model of Eisenman and Krasne [15], noting that the snug-fit mechanism assumes that the "*narrow part of the channel is a barrier to sodium because the dipoles (carbonyl groups) of the wall are held rigidly at the diameter of a K^+ ion (2.66 Å) can cannot all approach the small Na^+ ion (diameter 1.90 Å) as closely as the dipole of water can in solution*", whereas in contrast, the concept of field strength of Eisenman and Krasne "*would be useful if the dipoles of the channel are free to move and can be pulled in by small ions and pushed back by large ones*". Detailed ion-flux experiments highlighted the high selectivity of K^+ channels [63] but could be consistent with both explanations and Hille concluded by saying: "*Both such a flexible channel or the rigid channel of Bezanilla and Armstrong seem consistent with available information*". Nonetheless, Hille emphasized that "*geometric factors alone do not account for the strong selectivity against the small Na^+ and Li^+ ions*", stressing that "*more work is always required to remove water from small cations that from large cations, selectivity favoring large cations must occur whenever the site does not provide a much stronger attraction for small cations than for large cations. The properties of such a site are summarized by "low field strength"*. Summarizing these ideas, Hille concluded by the statement: "*The hypothesis is offered that the narrowest part of the K channels is a circle of oxygen atoms about 3 Å in diameter with low electrostatic field strength*", which combined the main ideas into a plausible compromise.

After the fundamental contributions by Eisenman and Krasne [15], Hille [16], and Bezanilla and Armstrong [14], no novel concepts about ion selectivity emerged. Clearly, it was not possible to go any further without any specific information

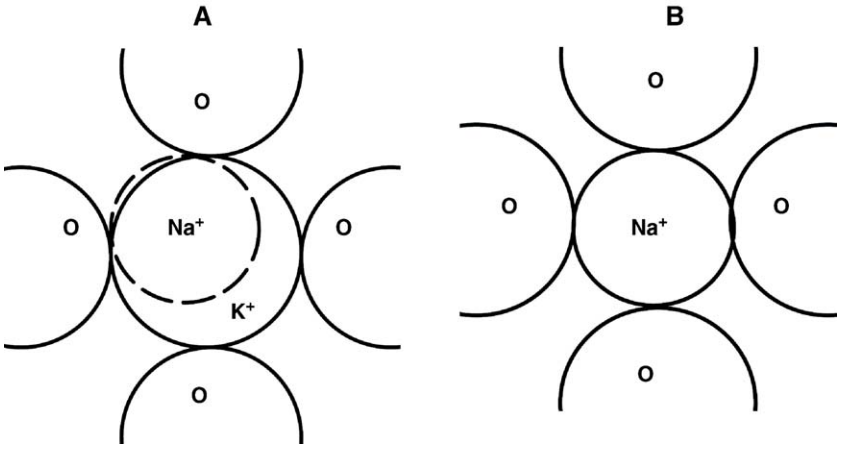

Fig. 5. (A) Simplified representation of a K^+ and Na^+ ions surrounded by 4 oxygen atoms of a proposed coordination cage in the narrowest part of K^+ pore. (B) Simplified representation of Na^+ solvation shell in water. Fig. 2b can be used to represent K^+ in the bulk as well [14].

about the three-dimensional structure of a K^+ channel. This took until 1998, when the crystallographic structure of the bacterial channel KcsA from *S. lividan* was determined at atomic resolution [3].

3.5. Crystal structure of the KcsA channel

The structure of the KcsA channel, shown in Fig. 1, is strikingly consistent with the classical views of a very selective, fast-conducting, multi-ion pore. The pore comprises a wide, nonpolar aqueous cavity on the intracellular side, leading up, on the extracellular side, to a narrow pore that is 12 Å long and lined exclusively by main chain carbonyl oxygens. Formed by the residues corresponding to the signature sequence TTVGYG, common to all K^+ channels [43], this region of the pore acts as a selectivity filter by allowing only the passage of nearly dehydrated K^+ ions across the cell membrane. The X-ray structure unambiguously demonstrated that the K^+ ion entering the selectivity filter have to lose nearly all their hydration shell and must be directly coordinated by backbone carbonyl oxygens. To explain how the pore was able to discriminate K^+ over Na^+, Doyle at al. [3] wrote "*The structure reveals that the selectivity filter is held open as if to prevent it from accommodating a Na^+ ion with its smaller radius. We propose that a K^+ ion fits in the filter precisely so that the energetic costs and gains are well balanced. The structure of the selectivity filter with its molecular springs holding it open prevents the carbonyl oxygen atoms from approaching close enough to compensate for the cost of dehydration of a Na^+ ion. The filter is constrained in an optimal geometry so that a dehydrated K^+ ion fits with proper coordination but the Na^+ ion is too small*", in close correspondence with the snug-fit mechanism of Bezanilla and Armstrong [44]. This simple and appealing structural mechanism was then widely adopted to explain the selectivity of the K^+ channel, as explicitly stated by several authors:

- "*A rigid K^+ pore, however, cannot close down around a Na^+ ion (0.95 Å), which does not bind snugly in the pore and thus has a much higher energy than in water*" [6].
- "*Each K^+ ion in the selectivity filter is surrounded by two groups of four oxygen atoms, just as in water: these oxygen atoms are held in place by the protein, and are in fact the backbone carbonyl oxygens of the selectivity filter loops from the four subunits. Furthermore, they solve the problem of stabilizing potassium in preference to sodium by precisely matching the configuration of oxygen atoms around a solvated potassium ion*" [7].
- "*…the filter, for structural reasons, cannot constrict sufficiently to bring more than two of the carbonyls within good bonding distance of the Na^+. As a result, the energy of the Na^+ in the pore is very high compared with its energy in water*" [8].
- "*…potassium fits optimally at these sites. While they expand to accommodate rubidium (easily) and cesium (at substantial energetic cost), they don't contract enough to cradle sodium*" [9].
- "*This filter…forms a narrow region of the channel that is lined by oxygen atoms and is sufficiently flexible to allow rapid hopping of ions between adjacent binding sites yet sufficiently rigid to allow discrimination between K^+ and Na^+ ions*" [10].
- "*…rigid 4-fold symmetry of the K^+ channel is solely optimized for K^+ ions, not for Na^+ ion*" [11].
- "*The channel pays the cost of dehydrating K^+ by providing compensating interactions with the carbonyl oxygen atoms lining the selectivity filter. However, these oxygen atoms are positioned such that they do not interact very favorable with Na^+ because it is too small. Because of its relative rigidity the channel would not afford favorable interaction with ions of with different than potassium radius*" [12].

The main idea is that the narrow pore is perfectly suited (at the sub-angstrom level) to provide a cavity of the appropriate size to fit K^+, but unable (for structural reasons) to adapt to the slightly smaller Na^+. This implies a significant structural inability to deform and adapt: the energetic cost upon collapsing to cradle a Na^+ (a structural distortion of about 0.38 Å) must give rise to a significant energy penalty (much larger than k_BT). Assuming the existence of molecular forces opposing a sub-angstrom distortion is tantamount to postulating structural rigidity. Furthermore, the geometry of such a rigid pore must be very precisely suited for K^+ because it would be unable to adapt (even by 0.38 Å) without paying a significant energy price (much larger than k_BT). Therefore, structural rigidity and geometric precision are two underlying microscopic consequences to the common view.

There are fundamental problems with the common view. Proteins, like most biological macromolecular assemblies, are "soft materials" displaying significant structural flexibility [45]. Despite some uncertainties, the B-factors of the KcsA channel indicate that the RMS fluctuations of the atoms lining the selectivity filter are on the order of 0.75 to 1.0 Å, in general agreement with numerous independent MD simulations of KcsA [18,20,28,46–57]. The magnitude of atomic thermal fluctuations is fundamentally related to the intrinsic flexibility of a protein, i.e., how it responds structurally to external perturbations [45]. These considerations suggest that, at room temperature, the flexible/fluctuating channel should distort easily to cradle Na^+ with little energetic cost, as is seen in MD simulations with Na^+ in KcsA [50,52,53]. The flexibility of the pore is further highlighted by the experimental observation that K^+ is needed for the overall stability of the channel structure [4,5,58–60]. Therefore, even ion channel proteins appear to be inherently too flexible to satisfy the requirement of the traditional snug-fit mechanism. Furthermore, structural flexibility is absolutely essential for ion conduction since in some places the diameter of the pore in the X-ray structure of KcsA (pdb id 1K4C) is too narrow (by ~1.0 Å) to allow the passage of a water molecule or a K^+ ion [45].

In a recent review, Gouaux and MacKinnon [61] discussed ion selectivity in the language taken from classical host/guest chemistry [62], stating "*The protein selects for a particular ion, Na^+ or K^+, by providing an oxygen-lined binding site of the*

appropriate cavity size". The main idea from host/guest chemistry, which bares many similarities with the snug-fit view [44], originated from the work of Pedersen and Frensdorff who showed an apparent relationship between cation diameter and crown ether hole-size [63]. However, further investigations indicated that significance of the "hole-size/cation-radius" relationship was quite limited. For example, Michaux and Reisse [64] noted that the enthalpies of binding between Na^+ and K^+ and the 12–18-membered crown rings did not correlate with the concept of hole-size, and concluded that "*…the thermodynamic study of complexation equilibria 1 and 2 shows that crown ether ring and cation sizes must be abandoned as correct predictor of the selectivity of crown ethers toward alkaline cations in solution*" [64]. Following this study, Gokel et al. showed that the flexible polyether systems did not abide by the hole-size rule [65], concluding that: "*the hole-size relationship probably plays its greatest role when the ligands are relatively inflexible*". In his extensive review, Dietrich said "*the hole-size/cation-diameter relationship is somewhat idealized (there are some large deviations)*" [62]. While the concept of a "hole" of a well-defined "size" ready to bind an ion of a specific radius can be understood in the case of a fairly rigid binding site, it more difficult to reconcile with the idea of a molecule that is highly flexible (see FAQ below).

4. Atomic level treatment of ion selectivity

4.1. Free energy perturbation molecular dynamics

For a complete description of selective ion conduction through the K^+ channel, both equilibrium and non-equilibrium aspects would need to be considered. Nonetheless, it is clear that the observed selectivity for K^+ arises primarily because the partitioning of Na^+ into the narrow pore is thermodynamically unfavorable (e.g., see the discussion of the punchthrough experiments above). Fundamentally, this implies that the relative free energy $\Delta\Delta G$ of K^+ and Na^+ in the pore and in the bulk solution,

$$\Delta\Delta G(K^+ \rightarrow Na^+) = [(G_{pore}(Na^+) - G_{bulk}(Na^+)) - (G_{pore}(K^+) - G_{bulk}(K^+))] \quad (3)$$

is larger than zero. According to electrophysiological measurements, $\Delta\Delta G$ is on the order of ~6 kcal/mol for K^+ channels. The key question about the selectivity of K^+ channels is to identify the physical origin of the unfavorable free energy $\Delta\Delta G$. Because of its smaller radius, the hydration free energy of Na^+ is ~18 kcal/mol more negative than that of K^+, i.e., $G_{bulk}(Na^+) \approx G_{bulk}(K^+) - 18$ kcal/mol. However, one may also note that $G_{pore}(Na^+) \approx G_{pore}(K^+) - 12$ kcal/mol, which implies that–in absolute terms–Na^+ in the pore is more strongly solvated than K^+ in the pore. This is one more indication that the selectivity filter is flexible and that the backbone carbonyl oxygens will coordinate a Na^+ transiently wandering through the pore. If the pore were structurally unable to distort, then any cation smaller or equal to K^+ would have almost the same solvation free energy. It is only when the relative hydration free energy is taken into account that the channel is selective for K^+ over Na^+.

Free energy perturbation (FEP) based on all-atom molecular dynamics (MD) simulations [66,67] represents the most fundamental approach to elucidate the microscopic origin of thermodynamic factors governing the function of biological systems. By carrying FEP simulations, it is possible to incorporate the effect of thermal fluctuations and the contributions from all the atomic coordinates into a computed free energy difference of interest. The difference in solvation free energy between K^+ and Na^+ can be expressed as [68]:

$$e^{-[G(Na^+)-G(K^+)]/k_BT} = \left\langle e^{-[E(Na^+)-E(K^+)]/k_BT} \right\rangle_{(K^+)} \quad (4)$$

where $E(Na^+)$ and $E(K^+)$ are, respectively, the potential energy with a Na^+ or a K^+ ion in the dynamical system (keeping all atomic coordinates unchanged). In the FEP expression, the bracket formally represents an average over configurations generated with a K^+ ion in the system

$$\langle A \rangle_{(K+)} = \frac{\int dr_1 dr_2 \cdots dr_n \; A \; e^{-E(K^+;r_1,r_2\ldots,r_n)/k_BT}}{\int dr_1 dr_2 \cdots dr_n \; e^{-E(K^+;r_1,r_2\ldots,r_n)/k_BT}} \quad (5)$$

(in practice, the total free energy difference between Na^+ and K^+ is computed by using a number of intermediate systems defined by a coupling parameter λ to join the two "end-points" [66,67]). Using the FEP method, the free energy difference between Na^+ and K^+ in the bulk solution [69,70] as well as inside the channel [20,28,46,56] can be calculated from all-atom MD simulations.

FEP simulations were performed for each of the five cation binding sites in the selectivity filter [20,28]. The calculations indicate that selectivity is not uniform along the pore. The most selective site is located in the middle of the pore (S_2). A similar trend was observed in the calculations by Luzhkov and Åqvist [56] using a different force field and methodology. Such variations in the free energy of selectivity as function of binding site were associated with the differences in hydration of the cation in the different binding sites. A cation in site S_2 is completely dehydrated and coordinated by 8 backbone carbonyl oxygens (from Gly77 and Val76). The result of the FEP calculations, with an unfavorable free energy of ~6 kcal/mol for Na^+ in the binding site S_2, are consistent with the experimental estimate deduced from the Ba^{2+} blockade by Neyton and Miller for the "lock-in" site [25,26] and the main features of the punchthrough experiments by Nimigean and Miller [27] (see also Fig. 2).

The results from FEP based on all-atom MD simulations shows unambiguously that the pore of KcsA can be selective for K^+ over Na^+, despite atomic fluctuations of the selectivity filter on the order of 0.5 to 1.0 Å RMS. The magnitude of the thermal fluctuations is illustrated in Fig. 6 with a superposition of instantaneous configurations. These results are relatively insensitive to changes in force field. The computed K^+/Na^+ selectivity for the most selective binding site of the KcsA (S_2) using different parameters/force-fields results in very similar trends (see Table 1). The computed selectivity is not a

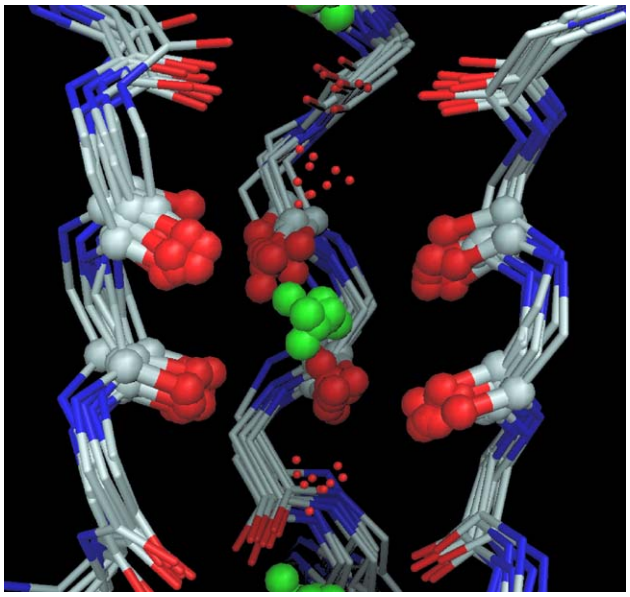

Fig. 6. Superposition of the frames from MD simulation [20] (green spheres are dynamics of K^+ ion in the binding site S_2, red dots are water molecules in binding sites S_1 and S_3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coincidental result for a given force field, but (perhaps surprisingly) a robust feature of the system.

4.2. Results from an exceedingly simple model

The FEP results from the realistic all-atom model of the KcsA channel embedded in a lipid membrane are consistent with experimental estimates. Accord with experiment is a prerequisite to go further with any analysis. But identifying the microscopic origin of robust ion selectivity despite significant atomic fluctuations and flexibility in an all-atom simulation (~40,000 atoms) is difficult. To this end, it is useful to examine the behavior of simpler systems [20]. Here, we consider a very simple "toy model" that comprises only 1 ion and 8 carbonyl-like groups freely fluctuating within a distance of 3.5 Å from the ion. The only positional restraint introduced in the minimalist model is a half-harmonic potential preventing the carbonyl oxygens from moving by more than 3.5 Å *away* from the ion. Of particular importance, no restraints are introduced to prevent the shell of carbonyls from shrinking and collapsing onto a small cation. This model is a caricature of reality intended to illustrate and capture some essential features of the real binding site S_2 in KcsA in the absence of any structural rigidity. By construction, there can be no structural rigidity in the model of freely fluctuating carbonyl groups. Yet, this model is nonetheless selective for K^+, with a $\Delta\Delta G$ on the order of ~6 kcal/mol as in the FEP calculations based on all-atom MD simulations. The selectivity is lost when there are only 6 freely carbonyl-like groups to coordinate the cation (decreases to ~2 kcal/mol). (The difference in hydration free energy between Na^+ and K^+ is still taken into account in these calculations.)

This result demonstrates that selectivity for K^+ over Na^+ can arise in such a simple system regardless of any architectural rigidity of the ligands about some average position. This view bare some similarities with the classical ideas of field strength and the thermodynamics analysis of Eisenman and Krasne [15] and are at the heart of the concept of "*Control of ion selectivity by electrostatic and dynamic properties of carbonyl ligands*", which led to the conclusion "*…selectivity in K^+ channels is primarily determined by the intrinsic physical properties of the ligands coordinating the cation in the binding site, rather than by the precise sub-angstrom geometry of the carbonyl oxygens lining a rigid pore*" [20]. Of course, this does not exclude additional contributions from the architectural rigidity of a binding site (see below).

What is the origin of K^+/Na^+ selectivity in this simple model? Expressing the free energy change in terms of differences in enthalpic and entropic components, $\Delta G = \Delta H - T\Delta S$, reveals that selectivity in the toy model is controlled by enthalpic factors. The dependence of the enthalpic (ΔH) and entropic ($-T\Delta S$) contributions as a function of the coupling parameter λ ($\lambda = 0$ and 1 corresponds to $K^+$and Na^+, respectively) is plotted in Fig. 7. The major determinant of the free energy difference between K^+ and Na^+ in the binding site is the enthalpic contribution ΔH. In contrast, changes in entropy between the Na^+ and K^+ bound states represent less than ~1 kcal/mol of the free energy difference. The enthalpic contribution corresponds to the average total potential energy in the system, which makes it particularly easy to interpret. The average potential energy comprises two opposing terms: the ion–ligand interaction, which favors a small cation, and the ligand–ligand interaction, which favors a large cation. In going from K^+ to Na^+, the change in ion–ligand attraction is about −18.6 kcal/mol, whereas the change in ligand–ligand repulsion is about +8.6 kcal/mol, yielding a favorable enthalpy of only −10.0 kcal/mol. The contribution from the ligand–ligand repulsion is, thus, essential to establish the selectivity for K^+ over Na^+. The influence of a secondary interaction such as the ligand–ligand repulsion on selectivity is reminiscent of the familiar concept of *strain energy* in host-guest chemistry [62]. However, while strain energy is traditionally associated with structural deformations of the host, in the present case it is seen to arise via "through-space" electrostatic interactions in the coordination shell of the cation.

Table 1
Free energy of K^+/Na^+ selectivity[a] ($\Delta\Delta G(K^+ \rightarrow Na^+)$) of S_2 binding site computed with different force-field parameters

	AMBER	GROMACS	CHARMM22	CHARMM27	CHARMM27′
G (kcal/mol)	4.77	3.5	4.98	5.89	5.32

AMBER [77]; GROMACS [56,78]; CHARMM22 [28,79]; CHARMM27 [80]; CHARMM27′ [20].

[a] FEP computations were performed on the KcsA channel embedded into the phospholipid membrane as described previously [20]. Prior actual FEP computations all simulated systems were equilibrated for 1000 ps starting from the structure reported at [20] using different force-field parameters in CPT ensemble at the temperature of 315 K. For each of the FEP computation the forward and backward directions free-energy perturbation ($K^+ \leftrightarrow Na^+$) had values of coupling parameter λ varying from 0 to 1 by 0.05 for a total 2.2 ns in simulation time.

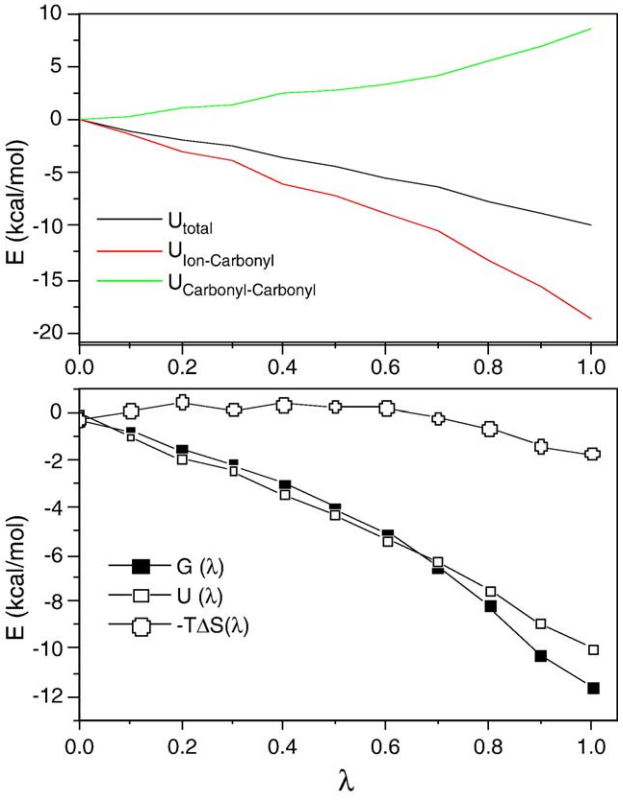

Fig. 7. Free energy decomposition as function of λ from the FEP simulations with the toy model consisting of 8 fluctuating carbonyl dipoles.

The importance of ligand–ligand repulsion was also noted previously by Luzhkov and Åqvist who stated "*it can be noted how the sites that are occupied by water molecules tend to 'swell' while those accommodating an ion tend to 'shrink', which illustrates the significant protein oxygen–oxygen repulsion intrinsic to the filter structure*" [56]. Even in simple toy models, altering the nature of the ligands can modulate selectivity. For example, as shown in Table 2, a binding site with four carbonyl groups and four water molecules can be favorable to Na^+. Expectedly, a binding site comprising only water molecules is not selective; the $\Delta\Delta G$ of transfer is −0.58 and 1.1 kcal/mol for 6 and 8 water ligands, respectively.

For obvious reasons, the magnitude of the repulsive interaction between two ligands coordinating an ion is sensitive to the electrostatic properties of the ligands. For example, two carbonyl groups on opposite sides of a Na^+ or a K^+ have an unfavorable interaction of about 4.3 and 2.9 kcal/mol, respectively (they are farther apart by ~0.8 Å when they coordinate the larger cation). This Na^+/K^+ difference of ~1.4 kcal/mol per carbonyl pair adds up to a significant number when there are 8 ligands around the cation (+8.6 kcal/mol in the toy model). In contrast, two water molecules or hydroxyl groups at the same position on opposite sides of a Na^+ or a K^+ have an unfavorable interaction of only about 1.0 and 0.7 kcal/mol, respectively. The large decrease in the repulsion upon substituting water molecules for the carbonyl groups has a very simple origin: the ligand–ligand repulsion varies essentially like the magnitude of the dipole squared, and the dipole of a carbonyl group is about twice the dipole of a water molecule. These findings impose constraints in the design of a binding site selective for Na^+. There may be several ways to create a binding site that is selective for Na^+ (see Table 2), but whether such a site could be formed exclusively by backbone carbonyl (C=O) groups seems unlikely. The high-resolution crystal structure for the sodium-selective leucine transporters determined recently [71] supports the conclusion from Noskov et al. [20]: the Na^+ binding sites are distinctively different from the 8-carbonyl ligands binding site observed in K^+ channels.

In conclusion, we find that it is the interplay of the attractive ion–ligand (favoring smaller cation) and repulsive ligand–ligand interactions (favoring larger cations) that govern size selectivity in a flexible protein binding site. Because such interactions can be directly modulated by the number and the type of ligands involved in ion coordination, altering the composition of the molecular groups forming a binding site appears thus to provide a very potent molecular mechanism to achieve and maintain a high selectivity in flexible proteins.

4.3. Interesting lessons from valinomycin

Valinomycin is a small cyclodepsipeptide that can catalyze the permeation of cations across lipid membranes [37,72]. It is highly specific for K^+ over Na^+ and its three-dimensional structure ion complex with K^+ has been determined using X-ray crystallography [73] (see Fig. 8 for illustration). For all these reasons, valinomycin has served over the years as a prototypical model system to formulate and test fundamental ideas about ion selectivity. Early studies examined the flexibility of the cyclic peptide [74]. Valinomycin has also been one of the first molecular system amenable to detailed MD simulation FEP studies of selective ion binding. Eisenman et al. explored the origin of ion selectivity and its sensitivity to the magnitude of the carbonyl dipoles [41,75]. They also examine the effects of increasing rigidity on the expected selectivity properties by applying harmonic constraints of varying magnitude. Additional simulation studies with explicit solvent (methanol) were done by Marone and Merz [76]. Generally, the FEP simulations displayed free energies favoring K^+, in good agreement with experiment. The issue of selectivity in valinomycin was revisited more recently by Noskov et al. [20], who performed computational experiments with explicit solvent (ethanol) in

Table 2
The variation of $\Delta\Delta G$ as a function of a toy-model ligand composition

Number of carbonyls	Number of water molecules	$\Delta\Delta G$ (kcal/mol)
8	0	6.2
7	1	4.79
6	2	2.28
5	3	−0.69
4	4	−2.11
6	0	3.40
5	1	3.19
4	2	0.26

The results are based on the FEP computations done on a simple model of one cation surrounded by eight carbonyl-like dipoles (comprising two atoms) with the oxygen atoms allowed to move freely within a sphere of radius 3.5 Å.

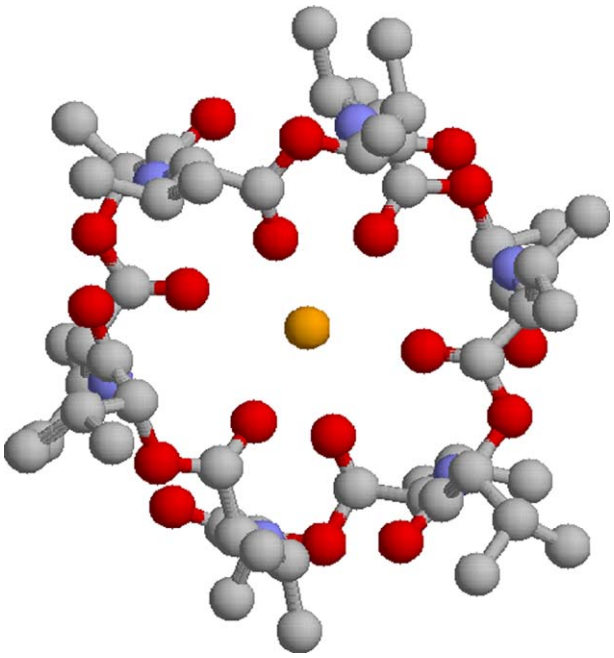

Fig. 8. Ball-and-stick molecular model of valinomycin bound to K^+ ion (orange) [73]. The cation is coordinated by 6 carbonyl groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which the carbonyl–carbonyl repulsion was artificially turned off. The FEP computations yield a free energy difference $\Delta\Delta G$ of 8.8 kcal/mol in favor of K^+ over Na^+. However, selectivity for K^+ decreased to 3.9 Kcal/mol when the carbonyl–carbonyl repulsion was turned off (decrease of 4.9 kcal/mol). This is in contrast with the results of a similar computational experiment performed on the KcsA channel, which showed that the pore became selective for Na^+ under the same conditions ($\Delta\Delta G$ changed from 5.3 kcal/mol to −2.9 kcal/mol). These computer experiments show that there is sufficient architectural rigidity in valinomycin to maintain some K^+ selectivity, even when the ligand–ligand repulsion is removed.

In contrast, selectivity cannot be preserved in KcsA under the same conditions because the pore is too flexible. This also explains how valinomycin succeeds in being selective for K^+ by providing only 6 donors coordinating the cation while the corresponding toy model with 6 freely fluctuating carbonyl-like ligands is only marginally selective for K^+.

The existence of some structural rigidity in a small ionophore such as valinomycin is not surprising. The molecule is a closed ring and each carbonyl group coordinating the cation is separated from the next by a small number of chemical bonds. Furthermore, a network of four intramolecular hydrogen bonds is formed in the outer region involved in cation coordination. All these factors contribute to confer sufficient rigidity (stiffness is probably a better word) to the molecule and increase its size specificity for K^+. In other words, the naturally "relaxed" conformation of the valinomycin molecule fits the size of K^+ very well and there is a small energy cost (strain) arising from the covalent forces (from bonds and angles) and intramolecular hydrogen bonds when it adapts to coordinate a cation that is smaller than K^+. Quantitatively, the loss of selectivity is 8.2 and 4.9 kcal/mol for KcsA and valinomycin, respectively. In comparison, the loss is 10.5 kcal/mol for liquid *N*-methylacetamide (NMA). On a relative scale of flexibility, valinomycin would be most rigid and KcsA is quite flexible (though less than liquid NMA). According to this analysis, the structural strain energy might be responsible for about half of the K^+ selectivity of valinomycin.

The example of valinomycin shows that the local covalent structures can act in concert to create stereospecific molecular fragments of sufficient local stiffness that are optimized to best bind cations of a certain size. Can one expect similar conditions to be met in the case of proteins? It may be possible to achieve some structural stiffness through tertiary packing motifs. However, this is difficult because the non-bonded interactions (van der Waals and hydrogen bonding) are relatively labile at room temperature. For example, the selectivity filter of KcsA is formed by the backbone from four independent subunits, which are not bound directly to one another, and the result is a fairly flexible (liquid-like) pore structure. Alternatively, it is possible to obtain stereospecific coordination with sufficient stiffness when two ligands are correlated locally via the covalent structure. For example, the backbone carbonyl and hydroxyl side chain of a threonine or serine, which are separated by only four chemical bonds (O=C–N–C_α–C_β–O_δ), can be configured as a stable elementary unit able to optimally coordinate a cation of a given size. Such a carbonyl backbone threonine side chain motif is observed in the case of the Na^+ binding sites of the leucine transporters [71].

5. Ion selectivity FAQ's

Based on our experience, a number of questions are frequently asked about the microscopic basis of ion selectivity, ion hydration, the significance of fluctuations, the importance of protein rigidity, etc… Answering those questions provides a good opportunity for clarifying a number of fundamental concepts and deepening our understanding of ion channels. We feel it is worthwhile to try and address here the most frequent questions.

5.1. The hydration free energy of Na^+ is about 20 kcal/mol more favorable than that of K^+. In this light, it would be amazing if K^+ channels were not selective since all the protein has to do is not to over-solvate Na^+ ions

The difference in hydration free energy between Na^+ and K^+ does indeed set a fundamental "baseline" for the function of all biological ion channels. This includes channels that are selective for K^+, as well as those that are selective for Na^+. Nonetheless, focusing exclusively on the difference in hydration free energy easily leads to oversimplifications (if differences in hydration free energy were the only important factor, then even a nonpolar carbon nanotube could be a highly selective K^+ channel!). For example, this argument overlooks that the selectivity filter is a highly electronegative environment, one that is very attractive for cations. Under physiological

conditions, the concentration of K^+ in the narrow pore is nearly 20 to 30 mol/l (2–3 ions in an effective volume of 100–150 $Å^3$). Only the repulsion between the K^+ makes rapid conduction possible. Paradoxically, one also may note that the interaction between a cation and a single backbone carbonyl is significantly larger than with a water molecule, and that this difference is even more prominent in the case of Na^+ than for K^+. In the language of Eisenman [17], carbonyls are "high field" ligands. Therefore, the real question should be how the protein succeeds in creating a highly attractive environment for K^+ without attracting Na^+.

5.2. Since the atomic thermal fluctuations take place at a rate several orders of magnitude faster than a single conduction event, how could they have any relevance to selectivity or permeation? Would not a permeating ion only "see" the average position of the atoms lining the pore?

This common argument about fluctuations, structural averaging, and ion selectivity [19] is at fault for two reasons. First, selectivity is governed by relative solvation free energies of ions, and in classical statistical mechanics (which is applicable to this situation), a free energy is a thermodynamic quantity independent of timescale. This is exemplified by considering Eqs. (2)–(4). Timescale, frequencies, and atomic masses simply do not appear in the mathematical expression for the free energy *G*. Second, the argument proceeds from confusion about the meaning of the averaging process. Even though any single individual fluctuation is of no particular significance, the cumulative averaging from a large number of thermal fluctuations gives rise to systematic statistical effects on the free energy that cannot be expressed (reduced) in terms of an average structure. In other words, the average energy cannot be deduced from the average structure. As a simple example, the average Coulomb interaction energy between two atoms is not the same as the Coulomb interaction energy between the two atoms standing at their average positions, i.e., $\langle 1/r\rangle \neq 1/\langle r\rangle$. For a K^+ in the KcsA channel, the approximation is invalid because the average distance is ~3 Å and the fluctuations are on the order of ~1 Å.

5.3. You showed that the free energy in a system of 8 freely fluctuating carbonyl-like groups is intrinsically selective for K^+ over Na^+. Does this imply that the three-dimensional structure of the channel is not important?

Of course not, this would be absurd! The selectivity for K^+ seen in the simple toy model with 8 freely-moving dynamical carbonyl-like groups signals the existence of an intrinsic propensity in such a system. By virtue of its local electrostatic properties, a solvation shell formed by 8 carbonyl-like groups is spontaneously selective for K^+ without any structural restraints. It is important to realize the power of the local propensity of the coordination shell. In the context of the three-dimensional structure of a correctly folded protein at room temperature like the KcsA pore (defined within ~1 Å, for scale of such motion see Fig. 6), this is probably the dominant factor. Nonetheless, mutations of residues that affect the stability of the folded protein may also have an impact on selectivity.

5.4. In the high resolution structure of the KcsA [3–5], the oxygen carbonyl oxygens of the binding sites clearly form a cavity of a size appropriate for K^+. Furthermore, the pore is distorted when K^+ is replaced with Na^+ [3–5]. Therefore, isn't selectivity simply arising because the protein provides a cavity of the appropriate size for K^+, but not for Na^+?

The "low-K" X-ray crystallographic structure, obtained with less than 10 mM concentration of K^+ and 250 mM concentration of Na^+, is indeed distorted relative to the conducting state. However, the significance of this structure is unclear because no Na^+ is detected in the electronic density. Furthermore, it was obtained under highly non-physiological conditions. Under normal circumstances, there is abundance of K^+ to occupy the pore and only 1 out of 1000 ions is a Na^+ wandering inside the selectivity filter. In this case, the structure of the selectivity filter is expected to remain close to the conducting conformation (if it were significantly distorted, then long blockades induced by external Na^+ would be detected experimentally). Such short-lived configurations with one Na^+ wandering inside the pore are akin to energetically unfavorable transition states, which are difficult to observe directly by X-ray crystallography. Nonetheless they can be characterized using all-atom MD simulations [50,52,53], which reveals that the selectivity filter is flexible ("liquid-like"), and that the carbonyl oxygens are able to adjust dynamically in order to form a cavity that is just of the appropriate size for Na^+. Therefore, the concept of a cavity of an appropriate size for K^+ cannot be invoked to explain why the free energy of the system is more favorable for K^+ than for Na^+.

6. Conclusion

Many of the key concepts in ion permeation were suggested several decades ago. The importance of hydration free energy and the need to compensate for dehydration upon entering a narrow pore was established already in the work of Mullins [13] and Bezanilla and Armstrong [14]. The availability of X-ray structures of K^+ channels at atomic resolution [3,4] gives us the unique opportunity to develop a rational quantitative view of the microscopic mechanism underlying ion selectivity. It is important to realize that, in order to understand ion selectivity, it is necessary to go beyond verbal assertions based on static structures. Selectivity in K^+ channels results from competing microscopic interactions and, ultimately, comes down to small free energy differences (a few kcal/mol). Assessing the relative importance of these interactions requires strict quantitative considerations based on accurate atomic models.

The present results show that the channel does not select for K^+ ions by providing binding sites of the appropriate cavity size. Selectivity for K^+ arises directly from the intrinsic local

physical properties of the ligands coordinating the cation in the binding site. The interplay between the attractive ion–ligand (favoring smaller cation) and repulsive ligand–ligand interactions (favoring larger cations) is the basic element that governs size selectivity in flexible protein binding sites. Such local interactions are directly modulated by the number and the type of ligands coordinating an ion. Altering the composition of a binding site, therefore, appears to provide a potent molecular mechanism to achieve and maintain a high selectivity in protein structures despite their significant conformational flexibility.

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