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ABSTRACT The fundamental biophysics underlying the selective movement of ions through ion channels was launched by George Eisenman in the 1960s, using glass electrodes. This minireview examines the insights from these early studies and the explosive progress made since then.

The recent passing of George Eisenman (December 18, 2013) inspired us to revisit the topic most associated with his passionate input, namely how the membrane proteins known as ion channels control passive movements of ions across biological membranes. Ion permeation has captivated biophysicists for more than half a century, and only now, with the combined advent of atomic-level structures and sophisticated computational wizardry, are the secrets of this amazing process beginning to be revealed. Why "amazing"? For example, because K^+-selective ion channels can discriminate between K^+ and Na^+ ions, which differ in radius by a mere 0.38 Ångstrom, and do so with 1000:1 reliability and at lightning speed near the diffusion limit, the dwell time of an ion in the pore of a channel is as fleeting as ~10^{-8} s. Understanding this remarkably-tuned process in K^+ channels requires attention to two perspectives: the ability of specific channels to discriminate between the ions they might encounter (i.e., selectivity); and the kinetics of ion movement across the channel pore (i.e., conduction).

The classical thermodynamic explanation of ion selectivity is that the relative free energy difference of ions in the pore relative to the bulk solution is the critical quantity to consider (1–4). Some of the earliest insights into thermodynamic selectivity derive from studies of ion binding to aluminosilicate glass electrodes (5,6). Depending on the composition of the glass, these electrodes, originally developed for their proton sensitivity, can exhibit a dramatic range of selectivities among the five alkali metal cations. In rank order, one might expect as many as $5 \times 4 \times 3 \times 2 \times 1 = 120$ different sequences of selectivities among these five cations. Remarkably, however, in the vast literature of selectivity in biological membranes, typically only 11 sequences are observed (with some exceptions). These became known as the "Eisenman sequences". The exact same selectivity sequences are observed in glass electrodes of various compositions.

Why are the free energy differences the way they are for a given system? To answer this question, one needs a physical mechanism. For Eisenman, numerical calculations stood as a critical component of the process of better understanding Nature. In other words, proposing a physical mechanism that is qualitatively reasonable is not enough—one must also test it by constructing atomic models leading to actual quantitative predictions (Fig. 1). In the early days, the concept of the anionic field strength of a binding site was formulated and tested with direct calculations based on exceedingly simple atomic hard-sphere models of ions, water molecules, and coordinating ligands such as shown in Fig. 1 *A* (2,5). Remarkably, these simple calculations led to the Eisenman selectivity sequences. Eisenman was able to account for the limited class of sequences by considering the equilibrium binding of cations to the glass, and the energetic competition between water and glass for the ions. The critical factor that determines the selectivity sequence of a given glass is the anionic field strength of the binding site on the glass. Briefly, the smallest group Ia cation, Li^+, holds water most tenaciously, so it will only dehydrate and bind in the presence of a strongly negative electrostatic potential.

By contrast, the largest cation, Cs^+, holds water least tenaciously. It cannot bind readily to a strongly negative site because the site itself greedily clings to water molecules, and thus prevents Cs^+ binding. However, Cs^+ is more willing, relative to the smaller cations, to dehydrate and bind in the presence of a weakly negative electrostatic potential. At the extremes, the highest anionic field strength glass shows a selectivity sequence of

$$Li^+ > Na^+ > K^+ > Rb^+ > Cs^+ \text{ (sequence XI)},$$

and the lowest anionic field strength glass shows a selectivity sequence of

$$Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \text{ (sequence I)}.$$

A very simple model, based on the relative Gibbs' free energies of binding and hydration, explains why there are only 11 sequences (5–7). The critical factor underlying the pattern of these selectivity sequences is that the "ion-site

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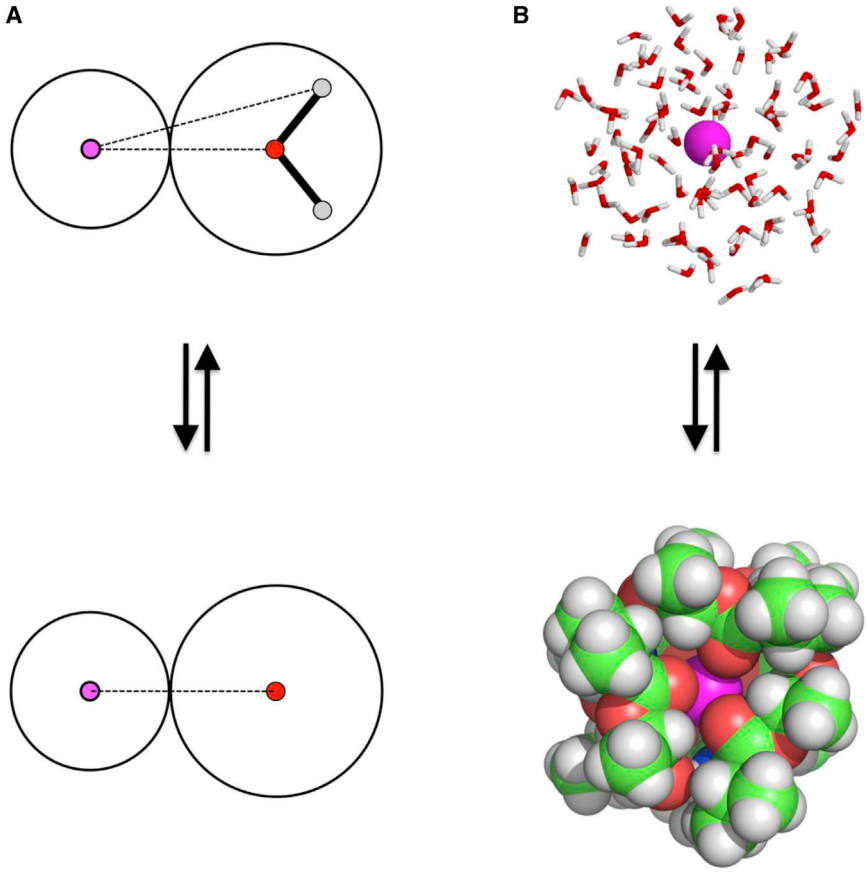

FIGURE 1 Structural models used in theoretical studies of ion selectivity. (*A*) Simple model used to introduce the concept of field strength leading to 11 cationic selectivity sequences (2,5,6). Ions, water, and ligands are represented by simple hard-spheres with embedded point charges. Selectivity arises from the difference in the interaction energy of the cation with a water molecule (*top*) and an anionic coordinating ligand (*bottom*). (*B*) Ion-selective transfer process is depicted with atomic models incorporating all molecular details in the case of solvation in liquid water (*top*) and binding to the K^+-selective ionophore valinomycin (*bottom*). Such atomic models were used to carry out some of the earliest MD free energy simulations on ion binding selectivity (12,13,15).

interaction energies fall off as a function of cation size as a lower power of the cation radius than do ion-water interaction energies" (5,6). The icing on the cake is that ion selectivity of channels in membranes appears to follow similar principles (7). The thermodynamic principles are evidently analogous. Moreover, Eisenman's contributions went far beyond the monovalent cation selectivity of potassium channels. His theoretical approach was seminal in understanding both cation and anion selectivity in a diverse range of physical and biological systems (8,9).

The advent of molecular dynamics (MD) simulations around this period was of critical importance to the field. This made it possible to construct increasingly realistic models of proteins (10), including ion channels (11), and examine the ion selectivity of carriers using the alchemical free energy perturbation (FEP) technique (12,13). With no experimental structures yet available for the ion-selective regions of biological K^+ channels, an important step forward was Eisenman's realization that other ion-selective systems could be used to computationally test the structural basis of his selectivity theory. Both peptidelike small ionophores, such as valinomycin and nonactin, and the ion-coordinating fivefold symmetry sites in icosahedral virus structures, thus caught his attention (13). As it turned out, these types of structures were indeed very relevant for the selectivity problem, because K^+-channel filters were eventually shown to be lined likewise by carbonyl groups (14). With the crystallographically determined valinomycin structure at hand, its selectivity could be energetically analyzed by atomistic computer simulations, as illustrated in Fig. 1 *B* (12,15). The anionic field strength (represented by the carbonyl ligand dipole moment) could then be varied artificially, and the successive progression through the different selectivity sequences, as a function of field strength, directly observed. Likewise, Eisenman and Alvarez (13) made computational predictions for the binding energetics and selectivity of the Ca^{2+} binding site at the fivefold symmetry axis of satellite tobacco necrosis virus, and they subsequently showed experimentally that this binding site had a marked rare-earth ion size selectivity (16). To this day, the general computational FEP/MD framework based on equilibrium thermodynamics used in these studies continues to be a critical tool to understand ion channels (17), transporters (18), and pumps (19).

Despite these early insights, it was always clear to Eisenman that explanations of selectivity solely based on thermodynamic equilibrium were too simple to account for the detailed properties observed in biological systems. Since the halcyon days of equilibrium binding studies on glass electrodes, the permeation landscape presented by the pores of ion channels has emerged as richer than anticipated. One important realization is that binding and

conduction of ions through a channel may act as contradictory processes, because although an ion has to leave the comfort of its hydration shells to selectively enter the mouth of a channel pore, if it binds the channel too tightly, it cannot move rapidly through it. This mini-conundrum is most apparent, perhaps, for K^+-channels, which attract K^+ ions much more forcefully than Na^+ ions, yet conduct K^+ ions much faster than Na^+ ions.

Another factor evident in early studies of permeation is that ions encounter a series of obstacles (i.e., energy barriers) and binding sites (i.e., energy wells) as they wend their way through the pore. One approach to understanding permeation is to consider that ions hopscotch from one well to the next over a series of barriers. When the number of barriers is rather limited, say <5, one can use so-called "rate theory" (20) to analyze and formulate the free energy profile experienced by an ion crossing the membrane. Hille (21) proposed that selectivity derives largely from the selectivities of the barriers, not the wells. Eisenman and Horn (7) later considered the possibility that binding sites and barriers within a particular channel might have different selectivity sequences. For example, if a channel presents two barriers, one of which has selectivity sequence I and the other has selectivity sequence XI, the channel as a whole will have an intermediate selectivity sequence that is not an Eisenman sequence at all. Rather, it is a so-called "polarizability sequence" (7). Interestingly, contemporary studies indicate that successive binding sites along K^+-selective channels display different selectivities (22). Another concept based on Eyring barrier models is that the energy levels for wells and barriers may not be static, and may therefore fluctuate on a timescale relevant to ion permeation (23). Finally, the biophysics of ion permeation and later structural studies show that multiple ions may cohabit the same channel simultaneously, and the interactions among these ions have profound consequences for ion conduction and selectivity.

Fast forward to the 21st century: atomic-level structures and all-atom simulations seem to have blown the permeation field wide open, as suggested by recent reviews (24–27). Once the KcsA channel structure was solved (14), the structural origin of K^+-ion permeation could finally be addressed by computer simulations of the "real structure" and a number MD simulation studies provided novel insight (22,28–31). Needless to say, George Eisenman took great interest in these simulations even though he had by then retired. Also, in the case of KcsA, the initial work largely revolved around calculations of equilibrium ion binding and selectivity, barrier heights, and energy landscape mapping (22,31), because direct all-atom simulations of spontaneous permeation were not possible. However, the general type of knock-on mechanism with multiion occupancy of the channel selectivity filter, involving key distinct states (22,31), and a surprisingly flat energy landscape (22), appear to be robust features of these channels.

Even with the advent of MD simulations, the concept of field strength has kept its relevance. For example, the selectivity filter in MD simulations of the KcsA channel displayed a range of atomic flexibility that seemed somewhat shocking at the time because a traditional host-guest mechanism of selectivity would require a fairly rigid cavity-size. Yet, free energy computations indicated that this was not strictly necessary to establish the thermodynamic free energy differences needed to support ion selectivity (32). The resilience of Eisenman's ideas is not entirely surprising because, as foreseen early on by Bertil Hille (21), the concept of field strength remains "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".

Nevertheless, despite the exciting progress, the chapter on ion selectivity in K^+ channels is far from closed. Very recently, a number of studies have revealed some extremely intriguing multiion aspects of selectivity in K^+ channels that appear to stand squarely outside the realm of equilibrium thermodynamics. By examining the properties of MthK (33) and NaK (34) mutants, Liu and Lockless (35) and Sauer et al. (36) showed that the channel becomes K^+-selective only if there are four consecutive binding sites along the filter. This has culminated more recently with studies of two engineered mutants of the NaK channel, referred to as "NaK2K" and "NaK2CNG". According to reversal potential measurements from single-channel electrophysiology, the NaK2K construct is K^+-selective and the NaK2CNG construct is nonselective. Remarkably, despite being nonselective in ion permeation, the NaK2CNG filter displays an equilibrium preference for binding K^+ over Na^+, as indicated by measurements with isothermal titration calorimetry and concentration-dependent ion replacement within the filter observed through crystallographic titration experiments.

K^+-selective channels bind two or more K^+ ions in the narrow filter, whereas the nonselective channels bind fewer ions. Based on the crystallographic titration experiments, the NaK2K construct has two high-affinity K^+ sites whereas the NaK2CNG construct has only one K^+-selective site. These experiments show that both K^+-selective and nonselective channels select K^+ over Na^+ ions at equilibrium, implying that equilibrium selectivity is insufficient to determine the selectivity of ion permeation (35,36). The data indicate that having multiple K^+ ions bound simultaneously is required for selective K^+ conduction, and that a reduction in the number of bound K^+ ions destroys the multiion selectivity mechanism utilized by K^+ channels. Although these experimental results are intriguing, the underlying microscopic mechanisms remain unclear. The implication is that the multiion character of the permeation process must, somehow, be a critical element for establishing selective ion conduction through K^+ channels.

The progress made, and the challenges that remain, are perhaps best illustrated by returning to computational

studies of the simplest membrane spanning structure known, namely the gramicidin A channel. Before detailed studies of selectivity and conductance of K^+-channels were launched, computational work on ion conduction through membrane channels was largely focused on this simple channel (37–41). In this case the permeation selectivity was monotonically size-dependent (Eisenman sequence I) and, in this respect, less interesting than K^+-selective channels. However, from an energetic point of view it was puzzling how this single helical structure could yield free energy barriers low enough to permit high conductivity (7,42). Computer simulations of increasing complexity in this case established that the combined effect of several contributions to ion stabilization along the pore (from the protein, membrane, single-file waters, and bulk solution) indeed results in low barriers to permeation (11,39,40). Furthermore, the most realistic model comes in close agreement with experimental measurements (11,43), although it is clear that work is still needed.

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