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Computational Studies of Membrane Channels

Review

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The determination of the structure of several members of the K^+ channel and aquaporin family represents a unique opportunity to explain the mechanism of these biomolecular systems. With their ability to go beyond static structures, molecular dynamics simulations offer a unique route for relating functional properties to membrane channel structure. The recent progress in this area is reviewed.

Introduction

One of the most basic attributes of living organisms is the cellular membrane, the envelope that helps preserve the integrity of the cell. Sophisticated and complex molecular machineries are associated with the membrane to perform a wide range of physiological functions. Membrane channels, in particular, are highly specific proteins that facilitate and regulate the permeation of material in and out of the cell. For example, K^+ channels (see Figure 1A) allow the rapid passage of K^+ ions across the lipid bilayer at a throughput rate near the diffusion limit while being able to discriminate against Na^+ by more than a thousand-fold. Other membrane channels, the members of the aquaporin family (see Figure 1B), have evolved to facilitate the rapid permeation of small neutral molecules, some as ubiquitous as water or glycerol, but to exclude almost everything else including protons.

Membrane proteins are typically difficult to characterize structurally because the requirement for maintaining a membrane environment hinders purification and crystallization. The recent progress in the structural and functional characterization of several members of the K^+ channel (Doyle et al., 1998; Zhou et al., 2001; Jiang et al., 2002a, 2003; Kuo et al., 2003) and aquaporin (Preston et al., 1992; Murata et al., 2000; Sui et al., 2001; Savage et al., 2003; Fu et al., 2000) family provide us with a unique opportunity to explain the function of these biomolecular systems. Although the experimentally determined three-dimensional structure of membrane channels yields a wealth of information, membrane transport processes are intrinsically dynamical and theoretical considerations are necessary for understanding the underlying mechanisms of selection and conduction. Furthermore, the membrane is a disordered environment (see Figure 1) with strong electrostatic interactions and complex dynamical behavior that influences channel properties and needs to be described computationally since no genuine structure exists. With their ability to go beyond static structures and include the membrane environment, molecular dynamics (MD) simulations based on detailed atomic models (McCammon et al., 1977) are now playing an increasingly important role in shaping our view of how membrane channels carry out their function. MD simulations, together with a number of specific computational techniques (see Methods, below), offer a unique route for interpreting and relating functional measurements to a protein structure. This review aims at highlighting the recent progress in this rapidly developing area.

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Potassium Channels

More than fifty years ago, Hodgkin and Huxley demonstrated that the action potential in the squid axon was caused by voltage-dependent variations in Na^+ and K^+ membrane conductances (Hodgkin and Huxley, 1952). The ionic conductances are associated with highly specific membrane-spanning proteins, the Na^+ and K^+ channels. Without such channels, the lipid membrane would present a prohibitively large energy barrier to the passage of charged ions. Ever since the seminal work of Hodgkin and Keynes (1955), it had been known that K^+ channels were selective narrow pores able to simultaneously contain two to three K^+ moving in single file. The determination of the structure of the bacterial KcsA channel from *Streptomyces lividans* in 1998 by Rod MacKinnon and coworkers offered a first view of the general architecture of these proteins (see Figure 1A; Doyle et al., 1998). Because of its structural similarity with eukaryotic K^+ channels, investigations of KcsA are expected to help understand a large class of biologically important channels.

One of the most important mechanistic questions about K^+ channels asks how they are able to achieve a fast throughput rate and yet remain highly selective for K^+ over Na^+. Traditionally, the ability of these channels to conduct K^+ at nearly the diffusion limit has been described in terms of concerted multi-ion transitions, the "knock-on" mechanism (Hodgkin and Keynes, 1955) in which ion-channel attraction and ion-ion repulsion play compensating effects, as several ions move simultaneously in single file through the narrow pore. The X-ray structure of KcsA (Doyle et al., 1998; Zhou et al., 2001), which revealed the presence of multiple dehydrated K^+ ions coordinated by main chain carbonyl groups in a narrow "selectivity filter" formed by the residues corresponding to the signature sequence TTVGYG common to all K^+ channels, is completely consistent with these ideas (Figure 1A). The availability of atomic resolution X-ray structures (Doyle et al., 1998; Zhou et al., 2001), together with methodological advances in bio-

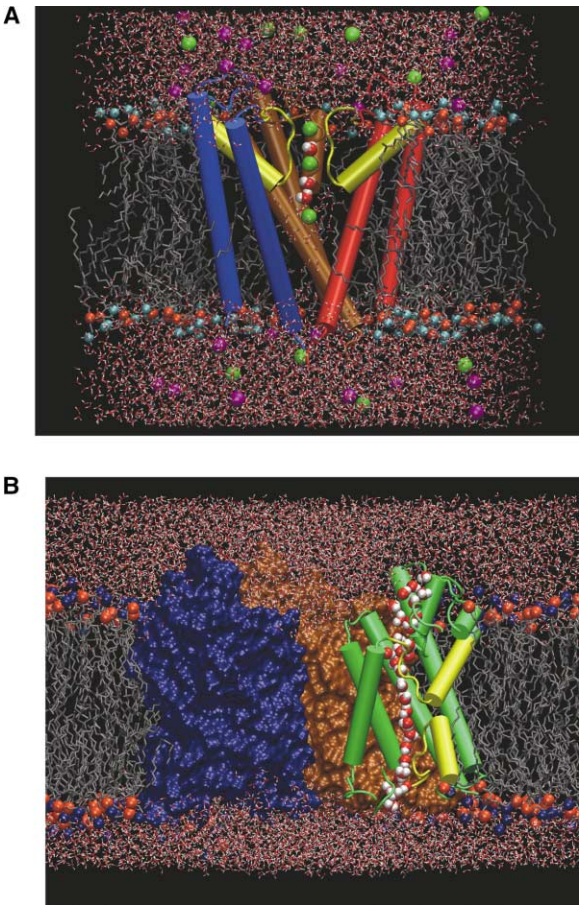

Figure 1. Atomic Models of Membrane Channels

Atomic models of the KcsA K^+ channel (A) (Bernèche and Roux, 2001) and aquaporin (B) (Tajkhorshid et al., 2002) in fully solvated phospholipid membrane for MD simulations (in both cases, one subunit is not shown to reveal the pore). The extracellular side is at the top and the intracellular side at the bottom. Distinctive architectural features of these membrane channels are the narrow single file pores and the α helices (yellow) running midway through the membrane. In the case of KcsA, these point directly toward the center of a wide nonpolar cavity on the intracellular side, leading up on the extracellular side to a narrow pore of 12 Å long occupied by K^+ (green) (Doyle et al., 1998), while they join at the NPA motif in the case of aquaporin, contributing to the overall hourglass shape of the pore with its approximate inversion symmetry. The atomic models of KcsA (Bernèche and Roux, 2001) (40,000 atoms) and aquaporin (Tajkhorshid et al., 2002) (100,000 atoms) were simulated with the all-atom CHARMM force field PARAM27 for proteins (MacKerell et al., 1998) and lipids (Schlenkrich et al., 1996) using the programs CHARMM (Brooks et al., 1983) and NAMD (Kale et al., 1999), respectively. The figure was drawn using VMD (Humphrey et al., 1996).

molecular simulations and the increase in computational power, made it possible to carry out theoretical studies of the K^+ conduction process.

Computations helped complement and reinforce the conclusions from experiments about the stable configurations of K^+ and water molecules inside the pore (Åqvist and Luzhkov, 2000; Bernèche and Roux, 2001). Using a systematic search procedure, the thermodynamical stability of all the possible ways to distribute any number of K^+ among the four cation binding sites located within the narrow selectivity filter (S_1, S_2, S_3, and S_4) was estimated using free energy perturbation (FEP, see Methods) (Åqvist and Luzhkov, 2000). It was concluded that only a small subset of occupancy states were energetically allowed with two K^+ separated by one water molecule occupying the selectivity filter, the so-called S_1-S_3 and S_2-S_4 configurations (Åqvist and Luzhkov, 2000). This important finding was later confirmed experimentally by high-resolution diffraction data of a mutant channel (Zhou and MacKinnon, 2004). Umbrella sampling MD simulations were used to compute the potential of mean force (PMF, see Methods), i.e., the multi-ion free energy landscape W (z_1, z_2, z_3) governing the movements of the K^+ ions along the pore axis (Bernèche and Roux, 2001). The calculation accurately reproduced the four K^+ binding sites in the pore of the KcsA channel that were already known (S_1 to S_4) and anticipated the existence of two additional sites located on the extracellular side of the channel (S_{ext} and S_0), which were revealed independently in high-resolution diffraction data (Zhou et al., 2001).

An extremely satisfying picture of the mechanism of ion conduction through K^+ channels has now emerged from the combined information of X-ray diffraction data and computations based on atomic models. The most fundamental mechanistic insight from MD simulations of KcsA is the concept of a single file of K^+ separated by water molecules dynamically moving through the narrowest region of the pore in a highly correlated fashion (Åqvist and Luzhkov, 2000; Guidoni et al., 1999; Bernèche and Roux, 2000; Shrivastava and Sansom, 2000), which is the rate-limiting step in the conduction mechanism (see also additional references in Roux, 2002). This is also the region where the driving force arising from the transmembrane electrostatic potential is predominant (Roux et al., 2000; Jiang et al., 2002b; Bernèche and Roux, 2003). The calculations suggest that the microscopic events leading to ion conduction occur according to a particular pattern, illustrated in Figure 2A, during which the five cation binding sites (S_0, S_1, S_2, S_3, and S_4) are transiently occupied by three ions (Bernèche and Roux, 2003). Such configurations, with alternatively two and three K^+ in the narrowest part of the pore, are qualitatively similar to the knock-on mechanism (Hodgkin and Keynes, 1955) and are consistent with ion occupancy determined by X-ray diffraction at high resolution (Zhou et al., 2001; Zhou and MacKinnon, 2004). One water molecule per K^+ is transported through the narrow pore, as observed experimentally. The maximum electric conductance of K^+ through the selectivity filter of KcsA (the g_{max}) was calculated using the multi-ion PMF (Bernèche and Roux, 2001) to be on the order of 300 to 500 pS (Bernèche and Roux, 2003), in remarkable agreement with experimental measurements (LeMasurier et al., 2001). Approximately one K^+ per 10–20 ns can be conducted through the channel under physiological conditions.

The computation of the multi-ion PMF helped to gain considerable insight into the strikingly delicate but effective energy balance underlying the knock-on mechanism. To allow rapid ion conduction, the strong attraction between the ions and the channel must be exquisitely compensated by the electrostatic repulsive forces be-

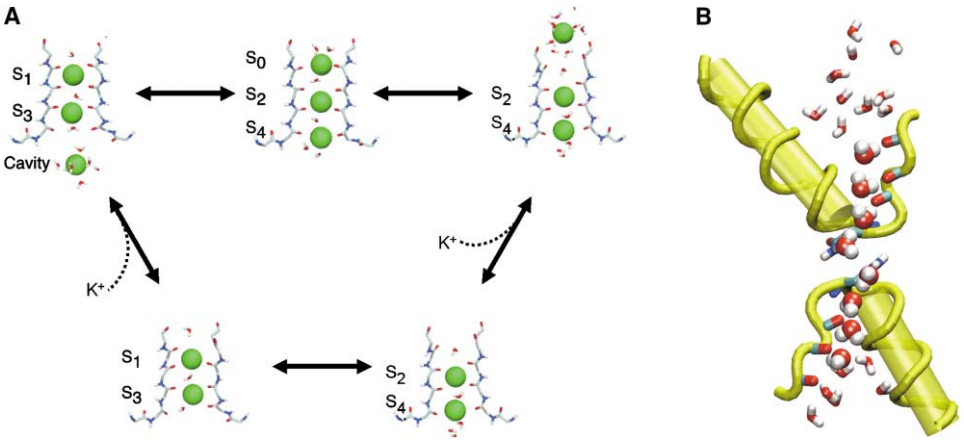

Figure 2. Single File Transport Mechanisms

Schematic illustration of the single file transport mechanism through the selectivity filter of KcsA (A) (Bernèche and Roux, 2003) and aquaporin (B) (Tajkhorshid et al., 2002). Ion conduction through KcsA (in the outward direction) is initiated when a K^+ hops in site S_4 from the intracellular cavity while two K^+ are located in the sites S_1 and S_3. This induces a concerted transition to a state with three K^+, occupying transiently the S_4, S_2, and S_0 binding sites, which is then followed by the rapid dissociation and departure of the outermost K^+ on the extracellular side. Water conduction through aquaporins is accompanied by a rotation of the water molecule at the center of the channel, where the two highly conserved NPA motifs meet to maintain the bipolar orientational ordering of the single file water molecules. The rotation of water dipole, enforced by electrical forces of the protein in a span of a few Å, is facilitated through hydrogen bond exchange between the two carbonyl arrays and the aspargine residues of the NPA motifs. (A) was drawn using DINO (Ansgar Philippsen, http://www.dino3d.org) and (B) was drawn using VMD (Humphrey et al., 1996).

tween the ions. The multi-ion PMF shows that knock-on is possible because the approach of an incoming ion on the intracellular side is not energetically prohibitive, while the dissociation of the outermost ion is accelerated by ion-ion repulsion (Bernèche and Roux, 2001). Electrostatic repulsion between the ions in the filter acts effectively at short distances in the complex environment of the narrow pore. Nonetheless, those repulsive forces are essential for rapid conduction; otherwise, the outermost ion would be tightly bound to its site and its exit toward the extracellular side would be opposed by a significant activation free energy. Atomic fluctuations of the protein are absolutely essential to achieve the rapid ion conduction, as there are regions of the selectivity filter that are effectively narrower than suggested by the van der Waals radius of K^+ and carbonyl oxygens (Åqvist and Luzhkov, 2000; Guidoni et al., 1999; Bernèche and Roux, 2000; Shrivastava and Sansom, 2000).

Having addressed questions about the rapid conduction of K^+, it is fundamental to understand how the channel remains selective for K^+ over Na^+. In its simplest terms, the observed selectivity arises primarily from the relative thermodynamic stability of different ionic species in the narrowest part of the pore and in the aqueous solution (Luzhkov and Åqvist, 2001; Bernèche and Roux, 2001). FEP computations (see Methods) showed that occupancy by Na^+ in the selectivity filter is thermodynamically unfavorable by 5–6 kcal/mol (Luzhkov and Åqvist, 2001; Bernèche and Roux, 2001). This suggests that K^+ is selected over Na^+ by a factor of several thousands, in good agreement with experiment (LeMasurier et al., 2001). Intriguingly, the magnitude of the thermal fluctuations of the backbone atoms forming the selectivity filter is large relative to the small size difference between Na^+ and K^+, raising fundamental questions about the mechanism that gives rise to ion selectivity. This paradoxical result suggests that the traditional explanation of ionic selectivity in terms of a rigid pore (Doyle et al., 1998) should be reexamined.

While MD and PMF computations provided much insight on the properties of ions inside the narrow selectivity filter, continuum electrostatic computations based on the Poisson-Boltzmann (PB, see Methods) equation helped clarify the consequences of the four α-helices (pore helices) with their COOH termini pointing toward the center of a 5 Å radius cavity at the level of the bilayer center (Figure 1A). Such an intriguing architectural feature is clearly the structural basis by which the channel overcomes the electrostatic barrier to ion translocation opposed by any low dielectric membrane (Doyle et al., 1998). More specifically, the PB calculations showed that the static and reaction fields in the channel were electrostatically tuned to exclusively stabilize a monovalent cation at the center of the cavity, thus revealing that the channel has the ability to exert a crude form of valence selectivity while keeping incoming cations fully hydrated (Roux and MacKinnon, 1999).

Finally, an important aspect of the function of K^+ channels concerns their ability to undergo conformational transitions in response to ligand binding or electrical signals leading to the opening or closing of the pore, a process generally referred to as "gating." Characterizing the molecular determinants of gating is a challenge, and despite the remarkable progress, numerous fundamental questions remain unresolved. Results from the bacterial channels provide some important clues. The full-length KcsA channel is mostly closed at neutral pH, but becomes more probable the open state at low intracellular pH (Heginbotham et al., 1999); the X-ray structures (Doyle et al., 1998; Zhou et al., 2001) (with its C-terminal truncated) correspond to the closed nonconducting state (Perozo et al., 1999; Roux et al., 2000). A similar

closed form is revealed by the X-ray structure of a bacterial inward-rectifier K^+ channel, the KirBac (Kuo et al., 2003). Data from electron paramagnetic spectroscopy (EPR) with site-specific spin labels suggest that channel gating involves the movements of the inner helices, which presumably lead to the opening of the pore on the intracellular side (Perozo et al., 1999). Molecular modeling and geometry optimization exploiting EPR data as constraints helped produce a model of the full-length closed KcsA channel in its membrane bound conformation (Cortes et al., 2001) and characterize the range of motion of the inner helices (Perozo et al., 1999). Furthermore, MD trajectories, biased to enforce an opening of the intracellular gate, revealed the propensity of the inner helices to bend near a highly conserved glycine residue (Biggin and Sansom, 2002). A similar helix bending is observed in the X-ray structure of the calcium-activated MthK channel (Jiang et al., 2002a), which was crystallized in its open state at high Ca^{2+} concentration. Whether such helical distortion is a completely general mechanism among K^+ channels is an unresolved question (del Camino et al., 2000; Johnson and Zagotta, 2001). Understanding the gating mechanism of voltage-activated channels is facing issues of even greater complexity. At the present time, the X-ray structure of a single voltage-gated bacterial channel, the bacterial KvAP, has been resolved (Jiang et al., 2003). However, it appears that the protein structure is distorted in the crystal and our knowledge of the native conformation of a voltage-gated channel remains incomplete. It may be hoped that atomic models exploiting all the information obtained from a wide range of structural, biophysical, and functional measurements will help develop a coherent perspective of channel gating in the future (Laine et al., 2004).

Water Channels

Aquaporins are abundantly present in all life forms (Heymann and Engel, 1999), for example, bacteria (Borgnia and Agre, 2001), plants (Javot and Maurel, 2002; Javot et al., 2003), and in the kidneys, the eyes, and the brain of humans. These proteins derive their name from their most prominent function as water channels across cellular membranes. But they can also conduct small molecules such as alcohols and even CO_2 (Uehlein et al., 2003) (but no ions). Their defective forms are known to cause diseases such as diabetes insipidus and cataracts (King and Yasui, 2002). These channel proteins, long suspected to exist, were discovered and characterized by Peter Agre and coworkers (Preston et al., 1992) in an experiment that demonstrated in a spectacular fashion how aquaporin 1 (AQP1) of red cells renders *Xenopus* oocytes more sensitive to osmotic shock. (AQP1 was initially termed CHIP 28 for "channel forming integral protein of 28 kDa.") Further work helped characterize the selective conduction of water through the *E. coli* channel AqpZ reconstituted in lipid bilayers (Pohl et al., 2001). A review of the cellular and molecular biology of aquaporin water channels is found in Borgnia et al. (1999).

Aquaporins are particularly intriguing membrane channels because of the apparent simplicity of their function: they transport water, but not protons. But they can also vary widely, including many different proteins in a single organism (e.g., 11 in human and more than 30 in plants), and can display multiple functions (e.g., transport of water as well as small linear alcohols like glycerol). As for the K^+ channel, a key question is how the protein manages to conduct water and other solutes rapidly and yet selectively. Further questions involve a rationalization of the differentiation of the channels, e.g., the AqpZ and GlpF channels in *E. coli* that serve for only water conduction and water as well as for glycerol conduction, respectively (Borgnia and Agre, 2001), of the 11 human aquaporins found in many tissues, or of the more than 30 plant aquaporins. A related question is why Nature chooses to utilize one and the same protein for both water and solute conduction, e.g., GlpF conducting water and glycerol, AQP3 conducting water and glycerol in human kidneys, or NtAQP1 conducting water and CO_2 in plants.

Research on aquaporins gained momentum through the elucidation of the structures of AQP1 (Murata et al., 2000; Sui et al., 2001), AQPZ (Savage et al., 2003), and GlpF (Fu et al., 2000). The path to these structures was arduous, involving several attempts to resolve the protein architecture of AQP1 by means of electron microscopy (Murata et al., 2000) and computational modeling (deGroot et al., 2001). Eventually, well-resolved crystallographic structures resulted that not only provided a detailed view of channel geometry and lining, but through detailed computational studies, also provided information about channel electrostatics (Tajkhorshid et al., 2002), energetics (Jensen et al., 2002), and dynamics (deGroot and Grubmuller, 2001; Tajkhorshid et al., 2002). In these studies, MD simulations turned out to be singularly successful in explaining the functions of aquaporins. This can be attributed to the fact that, first, conduction processes are by their nature dynamic and water and small solute conduction in aquaporins occurs extremely rapidly, namely on the nanosecond timescale that is covered by MD computations (deGroot and Grubmuller, 2001; Tajkhorshid et al., 2002), and second, aquaporins are actually relatively rigid and no sizable protein conformational changes accompany conduction events.

A unique architectural motif of aquaporins is their hourglass pseudosymmetric structure shown in Figure 1B, with two helices diving into the protein midway through the membrane and back to the aqueous phase in a distinct inverted helix fashion that exposes backbone atoms to the interior of the pore. The two helices are held in place at the channel center through two highly conserved NPA (asparagine, proline, alanine) motifs that also interact with channel water. The structure with its two inward pointed helices and approximate inversion symmetry brings about strong electrical fields along the channel axis that are inverted at the NPA motifs in a span of a few Å (Tajkhorshid et al., 2002; Jensen et al., 2003). MD simulations revealed that water and glycerol in GlpF is conducted along the channel by forming hydrogen bonds with the inverted helices backbone atoms and the NPA motifs (Jensen et al., 2001). The conducted glycerol is selected shape-wise by a selectivity filter lined by Phe, Trp, and Arg residues through a tight fit that

must be balanced energetically by hydrogen bonding between solute and channel to permit passage at low enough energy cost. Simulations showed that the diffusional motion of glycerol in the channel is driven by a fierce competition with channel water or hydrogen bonds to channel lining atoms, a competition that contributes to the effective diffusion coefficient of glycerol in the channel (Jensen et al., 2001). Interactive MD simulations (see Methods) revealed also that the electric field inversion at the NPA motifs electrostatically selects glycerol, since passage is energetically feasible only because of glycerol's ability to reverse the orientation of its OH dipoles by C-O bond rotations (Grayson et al., 2003), i.e., the molecule does not need to execute a sterically impossible rotation to invert its dipole moment.

Very fortunately, the speed of spontaneous, undirected water permeation crossing an entire channel is about one water molecule per nanosecond, such that it can be directly seen in MD simulations (deGroot et al., 2002; Tajkhorshid et al., 2002). Water transport can be induced either by osmotic pressure differences, giving rise to the osmotic permeability (P_f), or by a difference in tracer concentrations (e.g., heavy water), giving rise to the diffusion permeability (P_d) (Zhu et al., 2004). The latter accounts for water molecules transported through the entire channel, whereas P_f accounts for water entering and leaving the channel at its exits such that $P_f/P_d > 1$. In fact, the ratio P_f/P_d measures the number of essential steps that water molecules need to pass the channel, which is $N + 1$, where N is the effective number of water molecules arranged in singe file (see Figure 2B); N is calculated to be 11 and measured to be 12 (Zhu et al., 2004). The calculated P_f value is 7×10^{-14} cm^3/s in agreement with the experimental measurement (Zhu et al., 2004). For a pressure difference of 1 MPa, a flux of about ten water molecules per microsecond is predicted that corresponds well to physiological values. Glycerol conduction through GlpF is controlled by the PMF along the channel axis (see Methods). The rate of conduction can be estimated quantitatively from the mean passage time calculated using the PMF and the diffusion coefficient of the molecule along the pore axis as well as from more detailed modeling (Lu et al., 2003). At a periplasmic glycerol concentration of 0.5 M, one predicts a flux of 4×10^5 s^{-1}, which is within a factor two of the observed value (Lu et al., 2003). The PMF, which was computed using steered MD (see Methods) (Jensen et al., 2002), exhibits major barriers that coincide with the regions (selectivity filter and NPA motifs) where the channel develops its selectivity as described above; an energetically attractive periplasmic vestibule (Jensen et al., 2002) exhibits a well depth of about 4 kcal/mol that optimizes the glycerol conduction rate in balancing rates of attraction and escape (Grayson et al., 2003). The GlpF channel cannot yield directed glycerol transport under equilibrium conditions, but a combination of an asymmetric PMF with symmetric water pressure fluctuations at near microsecond frequency can lead to directed transport that is inward at low cellular glycerol concentrations and outward at high ones, turning GlpF possibly into a directed transporter (I. Kosztin, personal communication).

In the case of GlpF, the simulations showed nine single file water molecules along the narrowest part of the channel, predicted at locations that were verified crystallographically (Tajkhorshid et al., 2003). Water positions were also accurately predicted for a GlpF mutant (Tajkhorshid et al., 2003). Unexpectedly (though not in retrospect), the simulations revealed a bipolar orientational ordering of the channel water that matches the pseudosymmetric aquaporin architecture: from both ends of the channel, the water oxygens point into the channel, with two central water molecules pointing their oxygens sideways toward the two NPA motifs with which they strongly interact (Figure 2B; Tajkhorshid et al., 2003; Jensen et al., 2003). Detection of such water ordering falls below the currently achievable crystallographic resolution since only water oxygen can be resolved, but not water hydrogens. The bipolar arrangement is very pronounced; the simulations, by switching on and off various interactions, could establish that this arrangement is energetically enforced through water-water interactions, but also through the strong electrostatic field in the channel (Tajkhorshid et al., 2003). All subsequent simulations of aquaporins reproduced the bipolar water ordering.

The bipolar orientational ordering, which is maintained through channel electrostatics despite the rapid water conduction, has an important functional consequence. Normally, continuous single file water as formed in the channel (Figure 2B) conducts H^+ almost instantly via a Grotthus-like mechanism, i.e., they can function as a "proton wire" but only for a unidirectional water arrangement (Pomès and Roux, 1996). However, the bipolar ordering does not permit such conduction. Various calculations showed that proton conduction in aquaporin channels is indeed strongly inhibited on energetic grounds (deGroot et al., 2003; Chakrabarti et al., 2004; Burykin and Warshel, 2003; Ilan et al., 2004), the water ordering being a fingerprint of the energetics. Similar single file water conduction has been suggested to arise also through narrow channels formed by carbon nanotubes, and this has become the subject of intense theoretical study through the work initiated by Hummer (Hummer et al., 2001). Nanotubes are much simpler than aquaporins and, hence, significantly easier to comprehend, permitting a detailed analysis of water and proton transport. On the other hand, the more complex aquaporins can be used to highlight the design principles for optimized conduction and specificity.

Common Functional Principles

Although they differ fundamentally in their functional role, the members of the aquaporin and K^+ channels family display some common structural and dynamical features. As they do not appear to have any obvious phylogenic relation, it is likely that these features represent the result of convergent evolution. In both cases, the protein architecture exhibits a narrow pore, through which molecules and ions must move in single file. In both cases, α-helices are "interrupted" midway through the membrane, giving rise to specific electrostatic properties that play a functional role. A narrow channel, in which permeating species strongly interact with the atoms forming the "walls" of the pore, is essential for

selectivity, i.e., to recognize the correct substance and reject any other. The confined environment of such narrow pores is highly anisotropic and differs markedly from bulk solution. For this reason, considerations arising from atomic Å-scale interactions become absolutely essential for a valid explanation of membrane transport. Only MD simulations furnish the needed resolution today. It is particularly worth noting that many of the most distinctive characteristics of ions and of a single file of hydrogen bonded water molecules moving inside a narrow molecular pore were first revealed in a very influential MD study of the gramicidin channel carried out by Wilson and coworkers (Mackay et al., 1984) more than twenty years ago. The example of this visionary work is encouraging because it illustrates how a deep mechanistic insight can be achieved despite the limitations of computational models.

Both in the case of K^+ channels and aquaporins, the computations helped complement information from experiments about ion and water positions and water orientation. The successful prediction of binding sites for ions (Åqvist and Luzhkov, 2000; Bernèche and Roux, 2001) and water molecules (deGroot and Grubmuller, 2001; Tajkhorshid et al., 2002) along these narrow pores, as well as the accurate calculation of conduction rates (Lu et al., 2003; Zhu et al., 2004; Bernèche and Roux, 2003) in agreement with experiments demonstrate the ability of detailed atomic MD simulations to advance research on membrane channels. These results help reconcile the concept of favorable locations along a narrow pore (i.e., "binding sites") detected in X-ray structures, with the high throughput observed in functional measurements. Dynamical trajectories also permit an assessment of the functional importance of protein flexibility and thermal atomic fluctuations by making it possible to go beyond considerations based on static structures. For example, in places these pores are narrower than the van der Waals radius of K^+ or the shape of solutes, e.g., glycerol, showing that permeation would be completely impossible without thermal fluctuations. At this point, it seems clear that traditional concepts about ion selectivity based on a rigid pore (Doyle et al., 1998) are not compatible with our present knowledge about the flexibility and thermal fluctuations of proteins.

While atomic trajectories can be very informative, the most complete mechanistic insight about the function of K^+ channels and aquaporins is obtained from the calculated free energy landscape or PMF governing the key microscopic conduction steps. This makes it possible to estimate the rate of processes occurring on timescales that are not easily accessible from simple trajectories, e.g., the conduction of K^+ through KcsA (Bernèche and Roux, 2003) or the permeation of glycerol through GlpF (Lu et al., 2003). The PMF is not directly measurable and can only be "extracted" from detailed atomic calculations that have been thoroughly validated by comparison with experiments. Special simulation techniques are needed to calculate the PMF (see Methods). Simpler continuum electrostatic calculations based on the PB equation (see Methods) can also be very useful to illustrate fundamental principles in a particularly clear fashion. Such simple calculations highlighted the importance of desolvation penalty on the blockage of protons through the single file water wire (deGroot et al., 2003; Chakrabarti et al., 2004; Burykin and Warshel, 2003; Ilan et al., 2004) and showed that the pore helices of K^+ channels were tuned to electrostatically stabilize a monovalent cation in the central cavity. In both cases, PB calculations characterized long-range helix macrodipole effects in the low dielectric membrane environment.

What's Next?

Several factors have contributed to the apparent success of the computational studies on membrane channels: clearly posed conceptual challenges, suitability of simulation timescales, and opportunities for experimental verification. Membrane transport phenomena lend themselves to a formulation of very clear and precise mechanistic questions. There is a certain conceptual simplicity about membrane channels in contrast to most macromolecular biological systems. In particular, the reaction coordinate as a progression of ions or molecules along the axis of the pore is rather well defined. Furthermore, many membrane pores carry out their function passively, while undergoing only fast atomic thermal fluctuations (excluding gating). It is significant that those fluctuations, which are on the order of 1 Å, can be sampled accurately during the nanosecond trajectories and that they are accurately represented by atomic force fields. Importantly, computational tools and increased computational resources became available at the right time. While MD simulations of biological macromolecules have been around for nearly thirty years (McCammon et al., 1977), it is only in the last decade that sufficient computational resources have been available to permit the development of accurate atomic force fields, consistent treatment of long-range electrostatics, and an adequate sampling of realistic atomic models of these large systems (Tajkhorshid et al., 2003).

Success in future biomolecular computations seems to rely on the following ingredients: formulate and address clear mechanistic questions, identify the essential microscopic processes, construct realistic atomic models (including explicit solvent and membrane), generate sufficiently long MD trajectories to adequately sample the thermal fluctuations, and finally, characterize the free energy landscape governing the function by calculating PMFs along plausible reaction coordinates. The latter is a powerful approach to extend the results of MD to longer timescales. It is likely that the lessons drawn from these studies will help in the investigation of other channels, e.g., the ClC chloride channels, the mechanosensitive channels MscL and MscS, the voltage gating of K^+ channels, and macromolecular membrane complexes such as F_0-ATPase and membrane fusion proteins. One can anticipate that progress in understanding these complex systems will ultimately be achieved from a synergy between computational modeling and the information provided by a wide variety of experimental approaches.

Methods: Advanced Computational Techniques

Molecular dynamics (MD) constructs an atomic model of the macromolecular system, represents the microscopic forces with a poten-

tial function, and integrates Newton's classic equation $F = mA$ to generate a trajectory (McCammon et al., 1977). The result is literally a "movie" of the dynamical motions of all the atoms as a function of time. With the availability of potential energy functions for proteins (MacKerell et al., 1998) and lipids (Schlenkrich et al., 1996), as well as fast and reliable numerical algorithms, current MD methodologies have reached the point where one can generate trajectories of realistic atomic models of complex biological channel membrane systems (for a recent review of simulation methods, see Becker et al., 2001; Tajkhorshid et al., 2003). Simple MD trajectories, however, are somewhat limited in their ability to quantitatively characterize complex biomolecular systems, though their scope can be extended considerably with statistical concepts as well as advanced graphics and analysis methods, e.g., as furnished by VMD (Humphrey et al., 1996).

The free energy landscape, or potential of mean force (PMF), is a key concept for understanding the dynamics of microscopic processes. First introduced by Kirkwood (1935), it corresponds to the average reversible thermodynamic work function W done by the mean force $\langle F \rangle$ along a chosen reaction coordinate such as the position along the channel axis z, i.e.,

$$W(z_1) = W(z_0) - \int_{z_0}^{z_1} dz' \langle F(z') \rangle$$

(NB, the PMF can be generalized to multidimensional cases). Specific computational techniques have been designed to calculate the PMF from simulations (Torrie and Valleau, 1974; Jarzynski, 1997). In umbrella sampling, simulations are first performed in the presence of an external time-independent biasing potential to enhance the statistical sampling of the configurations of the system that are relevant (Torrie and Valleau, 1974). The bias introduced by this potential is then rigorously removed in postanalysis, thus enabling the characterization of the true unbiased free energy surface of the system (Ferrenberg and Swendsen, 1989). An alternative technique, steered MD (Jensen et al., 2002) and interactive MD (Grayson et al., 2003), is to use nonequilibrium calculation during which the system is submitted to an external time-dependent "pulling" biasing force. Again, the bias introduced by this perturbation can be rigorously removed in postanalysis (Hummer and Szabo, 2001; Park and Schulten, 2004) via a fundamental identity derived by Jarzynski (1997). Both techniques are powerful computational approaches to characterize quantitatively the free energy landscape governing the dynamics of complex biomolecular systems.

It is also possible to monitor the dynamical motions in the presence of imposed external forces to reproduce some aspect of the physical environment, such as membrane surface tension (Gullingsrud and Schulten, 2003), hydrostatic pressure gradient (Zhu et al., 2004), or transmembrane voltage (Tieleman et al., 2001). In some cases, MD trajectories including external pulling forces can even be generated interactively, e.g., to probe channel selectivity (Grayson et al., 2003). In addition, free energy perturbation (FEP) (Zwanzig, 1954; Kollman, 1993; McCammon and Straatsma, 1992), in which one molecular species is "alchemically" converted into another one using an altered potential (unphysical in its intermediate states), is an important technique to directly address questions about thermodynamic stability.

Lastly, approaches that are simpler and computationally less expensive than all-atom MD are very useful tools in studies of membrane channels. Macroscopic continuum electrostatic calculations based on the Poisson-Boltzmann (PB) equation, in which the polar solvent is represented as a structureless dielectric medium, can also help reveal the dominant energetic factors related to solvation (Roux et al., 2000). Simulations based on coarser representations that accelerate relaxation processes through smoothing interactions can help reduce the computational cost and enable the exploration of membrane phenomena occurring on long timescales (Lopez et al., 2004). Which approach is best to use depends on the microscopic detail of the system, the specific questions being asked, and the available computational resources. These resources, through computer clusters and suitable programs enabling highly parallelized calculations (e.g., NAMD [Kale et al., 1999]), have become available.

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References

Åqvist, J., and Luzhkov, V. (2000). Ion permeation mechanism of the potassium channel. Nature *404*, 881–884.

Becker, O.M., MacKerell, A.D., Roux, B., and Watanabe Eds, M. (2001). Computational Biochemistry and Biophysics. (New York: Marcel Dekker, Inc.).

Bernèche, S., and Roux, B. (2000). Molecular dynamics of the KcsA K(+) channel in a bilayer membrane. Biophys. J. *78*, 2900–2917.

Bernèche, S., and Roux, B. (2001). Energetics of ion conduction through the K^+ channel. Nature *414*, 73–77.

Bernèche, S., and Roux, B. (2003). A microscopic view of ion conduction through the KcsA K^+ channel. Proc. Natl. Acad. Sci. USA *100*, 8644–8648.

Biggin, P.C., and Sansom, M.S. (2002). Open-state models of a potassium channel. Biophys. J. *83*, 1867–1876.

Borgnia, M.J., and Agre, P. (2001). Reconstitution and functional comparison of purified GlpF and AqpZ, the glycerol and water channels from *Escherichia coli*. Proc. Natl. Acad. Sci. USA *98*, 2888–2893.

Borgnia, M., Nielsen, S., Engel, A., and Agre, P. (1999). Cellular and molecular biology of the aquaporin water channels. Annu. Rev. Biochem. *68*, 425–458.

Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., and Karplus, M. (1983). CHARMM: a program for macromolecular energy minimization and dynamics calculations. J. Comput. Chem. *4*, 187–217.

Burykin, A., and Warshel, A. (2003). What really prevents proton transport through aquaporin? Charge self-energy versus proton wire proposals. Biophys. J. *85*, 3696–3706.

Chakrabarti, N., Tajkhorshid, E., Roux, B., and Pomès, R. (2004). Molecular basis of proton blockage in aquaporins. Structure (Camb). *12*, 65–74.

Cortes, D.M., Cuello, L.G., and Perozo, E. (2001). Molecular architecture of full-length KcsA: role of cytoplasmic domains in ion permeation and activation gating. J. Gen. Physiol. *117*, 165–180.

deGroot, B.L., and Grubmuller, H. (2001). Water permeation across biological membranes: mechanism and dynamics of aquaporin-1 and GlpF. Science *294*, 2353–2357.

deGroot, B.L., Engel, A., and Grubmuller, H. (2001). A refined structure of human aquaporin-1. FEBS Lett. *504*, 206–211.

deGroot, B.L., Tieleman, D.P., Pohl, P., and Grubmuller, H. (2002). Water permeation through gramicidin A: desformylation and the double helix: a molecular dynamics study. Biophys. J. *82*, 2934–2942.

deGroot, B.L., Frigato, T., Helms, V., and Grubmuller, H. (2003). The mechanism of proton exclusion in the aquaporin-1 water channel. J. Mol. Biol. *333*, 279–293.

del Camino, D., Holmgren, M., Liu, Y., and Yellen, G. (2000). Blocker protection in the pore of a voltage-gated K^+ channel and its structural implications. Nature *403*, 321–335.

Doyle, D.A., Cabral, J.M., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., and MacKinnon, R. (1998). The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science *280*, 69–77.

Ferrenberg, A.M., and Swendsen, R.H. (1989). Optimized Monte-Carlo data-analysis. Phys. Rev. Lett. *63*, 1195–1198.

Fu, D., Libson, A., Miercke, L.J., Weitzman, C., Nollert, P., Krucinski, J., and Stroud, R.M. (2000). Structure of a glycerol-conducting channel and the basis for its selectivity. Science *290*, 481–486.

Grayson, P., Tajkhorshid, E., and Schulten, K. (2003). Mechanisms of selectivity in channels and enzymes studied with interactive molecular dynamics. Biophys. J. *85*, 36–48.

Guidoni, L., Torre, V., and Carloni, P. (1999). Potassium and sodium binding to the outer mouth of the K^+ channel. Biochemistry *38*, 8599–8604.

Gullingsrud, J., and Schulten, K. (2003). Gating of MscL studied by steered molecular dynamics. Biophys. J. *85*, 2087–2099.

Heginbotham, L., LeMasurier, M., Kolmakova-Partensky, L., and Miller, C. (1999). Single streptomyces lividans K(+) channels. Functional asymmetries and sidedness of proton activation. J. Gen. Physiol. *114*, 551–560.

Heymann, J.B., and Engel, A. (1999). Aquaporins: phylogeny, structure and physiology of water channels. News Physiol. Sci., *14*, 187–194.

Hodgkin, A.L., and Huxley, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation of nerve. J. Physiol. (Lond.) *117*, 500–544.

Hodgkin, A.L., and Keynes, R.D. (1955). The potassium permeability of a giant nerve fibre. J. Physiol. (Lond.) *128*, 61–88.

Hummer, G., and Szabo, A. (2001). Free energy reconstruction from nonequilibrium single-molecule pulling experiments. Proc. Natl. Acad. Sci. USA *98*, 3658–3661.

Hummer, G., Rasaiah, J.C., and Noworyta, J.P. (2001). Water conduction through the hydrophobic channel of a carbon nanotube. Nature *414*, 188–190.

Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. J. Mol. Graph. *14*, 33–38.

Ilan, B., Tajkhorshid, E., Schulten, K., and Voth, G.A. (2004). The mechanism of proton exclusion in aquaporin channels. Proteins *55*, 223–228.

Jarzynski, C. (1997). Non-equilibrium equality for free energy differences. Phys. Rev. Lett. *78*, 2690.

Javot, H., and Maurel, C. (2002). The role of aquaporins in root water uptake. Ann. Bot. (Lond.) *90*, 301–313.

Javot, H., Lauvergeat, V., Santoni, V., Martin-Laurent, F., Guclu, J., Vinh, J., Heyes, J., Franck, K.I., Schaffner, A.R., Bouchez, D., et al. (2003). Role of a single aquaporin isoform in root water uptake. Plant Cell *15*, 509–522.

Jensen, M.O., Tajkhorshid, E., and Schulten, K. (2001). The mechanism of glycerol conduction in aquaglyceroporins. Structure (Camb.). *9*, 1083–1093.

Jensen, M.O., Park, S., Tajkhorshid, E., and Schulten, K. (2002). Energetics of glycerol conduction through aquaglyceroporin GlpF. Proc. Natl. Acad. Sci. USA *99*, 6731–6736.

Jensen, M.O., Tajkhorshid, E., and Schulten, K. (2003). Electrostatic tuning of permeation and selectivity in aquaporin water channels. Biophys. J. *85*, 2884–2899.

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., and MacKinnon, R. (2002a). Crystal structure and mechanism of a calcium-gated potassium channel. Nature *417*, 515–522.

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., and MacKinnon, R. (2002b). The open pore conformation of potassium channels. Nature *417*, 523–526.

Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B.T., and MacKinnon, R. (2003). X-ray structure of a voltage-dependent K^+ channel. Nature *423*, 33–41.

Johnson, J.P., Jr., and Zagotta, W.N. (2001). Rotational movement during cyclic nucleotide-gated channel opening. Nature *412*, 917–921.

Kale, L., Skeel, R., Bhandarkar, M., Brunner, R., Gursoy, A., Krawetz, N., Phillips, J., Shinozaki, A., Varadarajan, K., and Schulten, K. (1999). NAMD2: greater scalability for parallel molecular dynamics. J. Comb. Chem. *151*, 283–312.

King, L.S., and Yasui, M. (2002). Aquaporins and disease: lessons from mice to humans. Trends Endocrinol. Metab. *13*, 355–360.

Kirkwood, J.G. (1935). Statistical mechanics of fluid mixtures. J. Chem. Phys. *3*, 300–313.

Kollman, P.A. (1993). Free energy calculations: applications to chemical and biochemical phenomena. Chem. Rev. *93*, 2395–2417.

Kuo, A., Gulbis, J.M., Antcliff, J.F., Rahman, T., Lowe, E.D., Zimmer, J., Cuthbertson, J., Ashcroft, F.M., Ezaki, T., and Doyle, D.A. (2003). Crystal structure of the potassium channel KirBac1.1 in the closed state. Science *300*, 1922–1926.

Laine, M., Papazian, D.M., and Roux, B. (2004). Critical assessment of a proposed model of Shaker. FEBS Lett. *564*, 257–263.

LeMasurier, M., Heginbotham, L., and Miller, C. (2001). KcsA: it's a potassium channel. J. Gen. Physiol. *118*, 303–314.

Lopez, C.F., Nielsen, S.O., Moore, P.B., and Klein, M.L. (2004). Understanding nature's design for a nanosyringe. Proc. Natl. Acad. Sci. USA *101*, 4431–4434.

Lu, D., Grayson, P., Tajkhorshid, E., and Schulten, K. (2003). Glycerol conductance and physical asymmetry of the *Escherichia coli* glycerol facilitator GlpF. Biophys. J. *85*, 2977–2987.

Luzhkov, V.B., and Åqvist, J. (2001). K(+)/Na(+) selectivity of the KcsA potassium channel from microscopic free energy perturbation calculations. Biochim. Biophys. Acta *1548*, 194–202.

Mackay, D.H., Berens, P.H., and Wilson, K.R. (1984). Structure and dynamics of ion transport through Gramicidin A. Biophys. J. *46*, 229–248.

MacKerell, A.D., Jr., Bashford, D., Bellot, M., Dunbrack, R.L., Evanseck, J.D., Field, M.J., Fischer, S., Gao, J., Guo, H., Joseph-McCarthy, D., et al. (1998). All-atom empirical potential for molecular modeling and dynamics studies of proteins. J. Phys. Chem. B *102*, 3586–3616.

McCammon, J.A., and Straatsma, T.P. (1992). Alchemical free energy simulation. Annu. Rev. Phys. Chem. *43*, 407.

McCammon, J.A., Gelin, B.R., and Karplus, M. (1977). Dynamics of folded proteins. Nature *267*, 585–590.

Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A., and Fujiyoshi, Y. (2000). Structural determinants of water permeation through aquaporin-1. Nature *407*, 599–605.

Park, S., and Schulten, K. (2004). Calculating potentials of mean force from steered molecular dynamics simulations. J. Chem. Phys. *120*, 5946–5961.

Perozo, E., Cortes, D.M., and Cuello, L.G. (1999). Structural rearrangements underlying K^+-channel activation gating. Science *285*, 73–78.

Pohl, P., Saparov, S.M., Borgnia, M.J., and Agre, P. (2001). Highly selective water channel activity measured by voltage clamp: analysis of planar lipid bilayers reconstituted with purified AqpZ. Proc. Natl. Acad. Sci. USA *98*, 9624–9629.

Pomès, R., and Roux, B. (1996). Structure and dynamics of a proton wire: a theoretical study of H^+ translocation along the single-file water chain in the gramicidin A channel. Biophys. J. *71*, 19–39.

Preston, G.M., Carroll, T.P., Guggino, W.B., and Agre, P. (1992). Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. Science *256*, 385–387.

Roux, B. (2002). Theoretical and computational models of ion channels. Curr. Opin. Struct. Biol. *12*, 182–189.

Roux, B., and MacKinnon, R. (1999). The cavity and pore helices in the KcsA K^+ channel: electrostatic stabilization of monovalent cations. Science *285*, 100–102.

Roux, B., Bernèche, S., and Im, W. (2000). Ion channels, permeation and electrostatics: insight into the function of KcsA. Biochemistry *39*, 13295–13306.

Savage, D.F., Egea, P.F., Robles-Colmenares, Y., Iii, J.D., and Stroud, R.M. (2003). Architecture and selectivity in aquaporins: 2.5 Å X-ray structure of aquaporin Z. PLoS Biol. 1(3): e72 DOI:10.1371/journal.pbio.0000072.

Schlenkrich, M.J., Brickmann, J., MacKerell, A.D, Jr., and Karplus, M. (1996). An empirical potential energy function for phospholipids: criteria for parameters optimization and applications. In Biological Membranes. A Molecular Perspective from Computation and Experiment, K.M. Merz and B. Roux, eds. (Boston: Birkhauser), pp. 31–81.

Shrivastava, I.H., and Sansom, M.S. (2000). Simulations of ion per-

meation through a potassium channel: molecular dynamics of KcsA in a phospholipid bilayer. Biophys. J. *78*, 557–570.

Sui, H., Han, B.G., Lee, J.K., Walian, P., and Jap, B.K. (2001). Structural basis of water-specific transport through the AQP1 water channel. Nature *414*, 872–888.

Tajkhorshid, E., Nollert, P., Jensen, M.O., Miercke, L.J., O'Connell, J., Stroud, R.M., and Schulten, K. (2002). Control of the selectivity of the aquaporin water channel family by global orientational tuning. Science *296*, 525–530.

Tajkhorshid, E., Aksimentiev, A., Balabin, I., Gao, M., Isralewitz, B., Phillips, J.C., Zhu, F., and Schulten, K. (2003). Large scale simulation of protein mechanics and function. Adv. Protein Chem. *66*, 195–247.

Tieleman, D.P., Berendsen, H.J., and Sansom, M.S. (2001). Voltage-dependent insertion of alamethicin at phospholipid/water and octane/water interfaces. Biophys. J. *80*, 331–346.

Torrie, G.M., and Valleau, J.P. (1974). Monte Carlo free energy estimates using non-Boltzmann sampling: application to the sub-critical Lennard-Jones fluid. Chem. Phys. Lett. *28*, 578–581.

Uehlein, N., Lovisolo, C., Siefritz, F., and Kaldenhoff, R. (2003). The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. Nature *425*, 734–777.

Zhou, M., and MacKinnon, R. (2004). A mutant KcsA K(+) channel with altered conduction properties and selectivity filter ion distribution. J. Mol. Biol. *338*, 839–846.

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

Zhu, F., Tajkhorshid, E., and Schulten, K. (2004). Theory and simulation of water permeation in aquaporin-1. Biophys. J. *86*, 50–57.

Zwanzig, R.W. (1954). High temperature equation of state by a perturbation method. J. Chem. Phys. *22*, 1420–1426.