Perspectives on: Conformational coupling in ion channels

Thermodynamics of electromechanical coupling in voltage-gated ion channels

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Membrane proteins are uniquely placed at the interface of the internal and external milieu of cells, and many of them unsurprisingly function as signal transducers involved in pathways critical for normal physiological activity. A majority of these membrane proteins have a modular architecture with distinct sensing and catalytic domains. Understanding how information flows from one part of the molecule to another is crucial for developing a detailed molecular-level understanding of their function. Voltage-gated ion channels are a class of such integral membrane proteins found ubiquitously in all kingdoms of life and are involved in both electrical and chemical signaling pathways (Hille, 2001).

Members of this superfamily share a common overall architecture wherein a functional unit is made up of four homologous domains arranged symmetrically around a central axis (Bezanilla, 2000; Long et al., 2005a). Each domain comprises six transmembrane segments and a reentrant pore loop, which forms the selectivity filter of the channel (Heginbotham et al., 1994). The segments S1–S4 constitute the voltage-sensing domain, which, as the name suggests, is the principal element for sensing changes in membrane potential (Bezanilla, 2000; Swartz, 2008). The exquisite voltage sensitivity of these proteins is, in large part, due to a distinct cluster of basic residues on the S4 segment (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). The four S5-S6 segments associate to form the functional pore domain through which ions can flux. The S6 segments line the ion permeation pathway, and, on the intracellular side, they come together at the bundle crossing to form the activation gate (Liu et al., 1997; del Camino et al., 2000).

Although significant progress has been made in developing a structural view of the gating process, the molecular driving forces that underlie these structural transitions remain poorly understood. Structures of channels in various states may suggest possible interactors, but experimental validation of these interactions is essential. In this perspective, we will principally focus on the thermodynamics of conformational coupling between the voltage-sensing domain and the gates in the pore domain, a process referred to as electromechanical coupling in recent literature. We will critically discuss the current state of knowledge and highlight the challenges in thermodynamic analysis of complex multistate proteins.

Defining "conformational coupling"

Dependence between two events can be conveniently expressed using conditional probabilities (Ben-Naim, 2010). For two events, A and B, where P(A) and P(B) are the respective probabilities of their occurrences, P(A∩B) is the probability that both A and B occur together, whereas P(A|B) is the conditional probability of event A, given that B has occurred. P(A|B) is expressed as:

$$P(A \mid B) = \frac{P(A \cap B)}{P(B)}. \quad (1)$$

In case the two events are independent of each other, P(A∩B) = P(A).P(B), and thus by Eq. 1, P(A|B) = P(A). For protein systems, the two events would represent conformational changes occurring in different parts of the protein. If the two conformational changes are independent of each other, then the conditional probability of each event will be equal to its innate probability (i.e., P(A|B) = P(A) and P(B|A) = P(B)), whereas if the two events are "coupled" (dependent), then the probability measures will be unequal. While mathematically sound and conceptually intuitive, such probability measures are seldom useful to describe the physical origins of dependence between two processes at the molecular level. Most often, to understand the molecular underpinnings of conformational changes within proteins it is necessary to quantify the dependence in terms of accurate and unambiguous energy measures.

Consider a simple case of two particles, where each can exist in two states, representing a structural unit of a protein capable of undergoing conformational

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transitions. Coupling between two such particles can be fully described by four state-dependent interactions: the particles can interact with each other when either of them are resting or activated (Fig. 1). This system can exist in four possible states, with two of them being the end states, namely, when both particles are in an activated or resting conformation. Thus, the net coupling between the two particles, θ, can be expressed as:

$$\theta = \frac{\theta_{RR}\theta_{AA}}{\theta_{RA}\theta_{AR}},$$

where θ is defined as the ratio between the "like" state interactions (θ_{RR}, θ_{AA}) and the "unlike" state interactions (θ_{RA}, θ_{AR}). In case the two particles are positively coupled, $\theta > 1$, and the net like state interactions will be stronger than the net unlike state interactions. Conversely, when they are "negatively" coupled, $\theta < 1$, the unlike state interactions will be greater than like state interactions, and the intermediate states will be stabilized. $\theta = 1$ implies that the two particles are not coupled to each other but does not necessarily mean that there are no underlying interactions between these particles. In theory, $\theta = 1$ simply indicates that the net like state interactions balance the unlike state interactions.

Thus, in terms of energetic effects, coupling by definition alters the stability of intermediate states. In addition, coupling could also contribute to the net free-energy difference between the two end states. For instance, let us consider the two-particle system described earlier but assume that the only interaction between them is θ_{AA} (i.e., when $\theta_{RA} = \theta_{AR} = \theta_{RR} = 1$). In this case, the presence of interaction alters the stability of the doubly activated state relative to the ground state. Functionally, this would be manifested as the difference in net free energy for activation along with corresponding change in forward and backward rates. If, however, the two structural units also interact with each other in the resting state and $\theta_{AA} = \theta_{RR}$, the interactions between the two particles does not produce a "net change" in the energy level of the final state (Fig. 1). In this scenario, conformational coupling is manifested only as a "kinetic effect" wherein the forward and backward rates are modified. Note that the unlike state interactions (θ_{RA}, θ_{AR}) never contribute to the net energy imbalance between the two end states even though they determine the coupling strength.

Experimental quantification of coupling interactions

To determine the origins of such coupling interactions at a molecular level, it is essential to measure interaction energies and identify perturbations, which alter coupling energies. Wyman in his influential studies on hemoglobin had shown that the Hill energy extracted from a logarithmic transformation of the direct ligand binding curve estimates the interaction energy between the ligand binding sites (Wyman, 1967). Horrigan and Aldrich (1999, 2002) have used similar strategies to estimate the coupling interactions between the different sensing and catalytic domains of the BK channel, although they considered a specific allosteric model to derive these relationships.

Most models of coupling use a single coupling parameter to describe energetic linkage between two structural domains of a protein. The six independent parameters in our initial model can be normalized to yield a model with three (normalized) energetic parameters, K_V, K_P, and θ (Fig. 2 A; Chowdhury and Chanda, 2010). It is to be noted that K_V and K_P may be different from the true intrinsic equilibrium constants of the voltage sensor and the pore, as they were obtained through normalizations that incorporate multiple state-dependent interactions.

Recently, we showed that for a generalized voltage-dependent allosteric system, the interaction energies associated with a specific structural unit can be extracted using an analytical technique, not limited by the size or symmetry-based constraints (Chowdhury and Chanda, 2010). The procedure, referred to as the

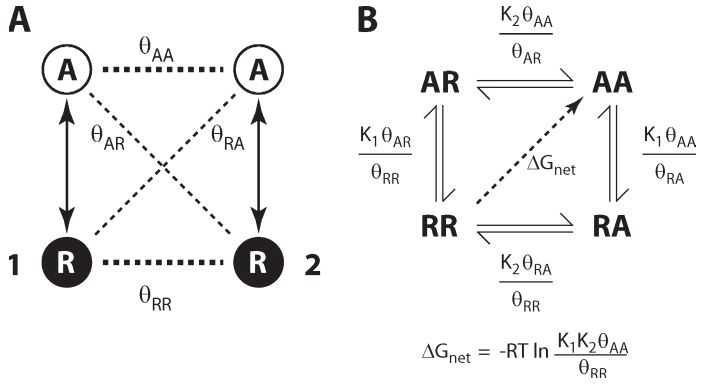

Figure 1. Definition of coupling. (A) Two particles, 1 and 2, each can exist in two states, R and A. Each of them have an intrinsic preference for one of the two states, which is determined by its intrinsic free-energy difference between the two states (represented by the solid arrows). There are four state-dependent interactions between them: the horizontal broken lines represent the "like state" interactions, θ_{AA} and θ_{RR}, which are the interactions when both particles are in the A or R states, respectively; the diagonal broken lines represent the "unlike state" interactions, θ_{AR} and θ_{RA}, which are the interactions when the particles are in dissimilar states. (B) The system in A is represented in a reaction scheme showing the transitions between the different states. Alongside each transition, its equilibrium constant is shown. K_1 and K_2 are the intrinsic activation constants of the two particles. The presence of the state-dependent interactions modifies each of the equilibrium constants, but the net free-energy difference (ΔG_{net}) between the doubly resting (ground) state, RR, and the doubly activated (final) state, AA, is determined by the intrinsic equilibrium constants and the like state interactions.

χ-value analysis, involves measuring the conformational transition of a particle, using a site-specific probe, over a wide range of voltages. When applied to the pore, we plot $\ln(P_O/1 - P_O)$ versus voltage (Fig. 2 B). At very low and high voltages, this plot will be linear with a slope proportional to the intrinsic voltage dependence of the pore. These asymptotes can be extrapolated to the V = 0 axis to extract the two intercepts, χ_- and χ_+. The difference between the two χ values, referred to as the χ^{diff}, is a measure of the interaction energy of the pore with all the other domains in the system. Indeed, for a general system consisting of N-interacting particles, the χ^{diff} for a particle is the ratio of all its like state interactions versus unlike state interactions and therefore is a direct measure of coupling interaction. This parameter is independent of the intrinsic stabilities of the different particles and is a linear correlate of the interaction energy of the particle with the remainder of the protein. To identify the specific residues of the protein, which mediate such interactions, one measures only the χ^{diff} parameter for different perturbations. Also, by measuring the effect of the perturbations on the χ_- and

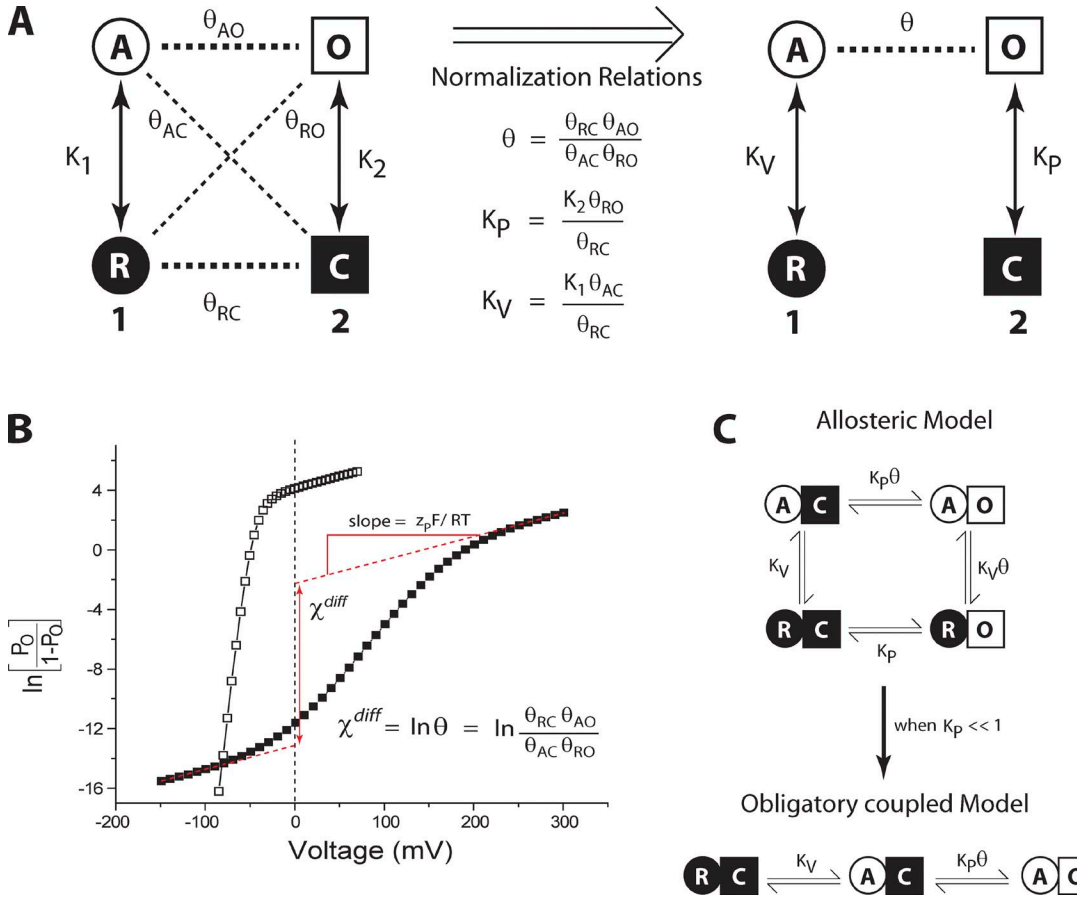

Figure 2. χ-value analysis for a voltage-dependent system comprising two particles. (A) The particle diagram for a system comprising a voltage sensor (which can exist in two states, R and A) and a pore (which exists in two states, C and O). K_1 and K_2 represent the intrinsic equilibrium constants of the voltage sensor and the pore in the absence of any other interactions. There are four state-dependent interaction terms between the two particles (θ_{RC}, θ_{AC}, θ_{RO}, and θ_{AO}; as in Fig. 1 A). The energetic parameters of this system can be normalized through the relations shown. The voltage dependencies of K_V and K_P can be expressed as: $K_i = K_i^0 \exp(z_i FV / RT)$ (i = V, P), where K_i^0 is the voltage-independent part of the equilibrium constant and z_i is its voltage dependence; F, R, and T represent the Faraday constant, the universal gas constant, and the temperature, respectively. Because the four coupling constants are voltage independent, K_V has the same voltage dependence as K_1, and K_P has the same voltage dependence as K_2. The coupling parameter in the normalized version, θ, is the ratio of the like state interactions ($\theta_{RC}\theta_{AO}$) and the unlike state interactions ($\theta_{AC}\theta_{RO}$). (B) Using arbitrary values of the energetic parameters, the $\ln(P_0/1 - P_0)$ vs. voltage plot for the pore was simulated for the allosteric model in A (closed symbols). The broken red lines are the extrapolations of the linear regimes (obtained at high and low voltages). The slope of the red lines is governed by z_P. The difference of the intercepts created by the linear extrapolations on the V = 0 axis is the χ^{diff} for pore, which is linearly proportional to the difference in the like state interaction energies ($-RT\ln(\theta_{AO}\theta_{RC})$) and the unlike state interaction energies ($-RT\ln(\theta_{AC}\theta_{RO})$). (C) The state diagram for an allosteric model comprising two particles, using the normalized parameters: there are four possible states of the system, depending on the conformations of each of the two particles. When K_P becomes very low, the allosteric model reduces to a linear sequential scheme representing an obligatorily coupled system. In terms of the un-normalized parameters, this could occur when $K_2 << 1$, $\theta_{RO} << 1$, and/or $\theta_{RC} >> 1$. The $\ln(P_0/1 - P_0)$ vs. V plot for the pore, for the obligatorily coupled model, shown in B (open symbols), keeps on decreasing steeply at hyperpolarizing voltages.

χ_+, useful information about the state dependence of the interactions can also be obtained.

The allosteric model will reduce to an obligatory coupled model if state-dependent coupling interactions are large or the intrinsic stability of the open pore (relative to the closed state) is low (i.e., $K_P << 1$; Fig. 2 C). In the allosteric scheme considered here, kinetic modeling can extract three independent equilibrium constants, which are sufficient to describe the energetics of the coupled system. However, kinetic analysis of obligatory systems will yield only two independent equilibrium terms, and consequently, the interaction energy cannot be independently estimated. Furthermore, the $\ln(P_O/1 - P_O)$ versus voltage plots (Fig. 2 B) show that, for obligatorily coupled systems, the steepness of the curve is characteristically large at hyperpolarized potentials and is not equal to that at depolarized potentials. Thus, it is not meaningful to extract the χ-value for obligatorily coupled systems.

Obligatory and allosterically coupled voltage-sensitive ion channels

The voltage-dependent gating behavior of the BK channels can be mostly accounted by a Monod-Wyman-Changeux model (MWC)-like allosteric model (Cox et al., 1997; Cui et al., 1997; Horrigan and Aldrich, 1999; Horrigan et al., 1999). According to these models, the pore domains can open even when the voltage sensors are not activated, albeit with a low probability. Activation of the voltage sensors makes the open pore progressively more stable, resulting in large open probabilities once the voltage sensors activate. A similar allosteric coupling model has been envisioned for HCN (Altomare et al., 2001; Chen et al., 2007; Kusch et al., 2010; Ryu and Yellen, 2012) and KCNQ channels (Osteen et al., 2010, 2012). In contrast, the coupling between voltage sensor and pore is obligatory in voltage-gated potassium and sodium channels, such that the pore domain cannot open until the voltage-sensing domains have transferred all their charges (Hirschberg et al., 1995; Islas and Sigworth, 1999).

Similar to the plots shown in Fig. 2 B, allosteric and obligatory behavior can be distinguished by plotting the $\ln P_O$ with respect to voltage. As shown originally by Almers (1978), in a linear activation scheme involving a single open state, the slope of $\ln P_O - V$ curves increases upon hyperpolarization and reaches a limiting value (Schoppa et al., 1992). This value corresponds to the total gating charge associated with channel opening. It should be noted that further hyperpolarization is unlikely to change this limiting slope value because all the gating charges have retracted to their resting state conformation, as reflected by the saturation of Q-V curves (Seoh et al., 1996; Sigg and Bezanilla, 1997; Islas and Sigworth, 1999). The implication of this observation is that the pore opening always requires prior activation of the voltage sensors. In contrast to the voltage-gated ion channels, the slope of the $\ln P_O - V$ curve for BK channels initially increases but upon further hyperpolarization decreases to a value less than the total gating charge (Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999; Ma et al., 2006). This indicates that the pore domain has some intrinsic voltage sensitivity, and at very highly hyperpolarizing voltages (where $P_O = \sim 10^{-7}$), the pore is able to open without the voltage sensor activating.

Molecular determinants of electromechanical coupling

Although it has not been feasible to obtain a direct estimate of the magnitude of interaction energy between the voltage sensor and pore in an obligatorily coupled voltage-gated ion channel, in this section we will consider various strategies that have been applied to identify the molecular mechanisms of this coupling. The search for molecular determinants of electromechanical coupling have to a large extent focused on a region of the polypeptide chain, known as the S4-S5 linker, that covalently links the voltage sensors to the pore domain. Initial evidence of the role of this region in coupling came from studies on a leucine heptad motif found in this region, which when perturbed results in large modification of channel gating (McCormack et al., 1991, 1993). In a remarkable experiment, Lu et al. (2002) were able to confer voltage sensitivity to channels that natively feature only the pore domains by fusing them to the voltage-sensing domains derived from the Shaker potassium channels. They showed that complementarity of the S4-S5 linker region with the C-terminal end of the S6 transmembrane segment was crucial for cross-talk between the voltage sensor and pore.

Various perturbation studies have examined the functional effects of mutations in the S4-S5 linker regions on the voltage-dependent opening in different members of this superfamily (Sanguinetti and Xu, 1999; Chen et al., 2001; Tristani-Firouzi et al., 2002; Decher et al., 2004; Ferrer et al., 2006; Soler-Llavina et al., 2006; Labro et al., 2008; Muroi et al., 2010; Van Slyke et al., 2010; Labro et al., 2011; Wall-Lacelle et al., 2011). In many early studies, attributes such as rightward shifts in conductance-voltage curves, slowed kinetics of channel opening, and increased deactivation rates were used to identify sites involved in coupling voltage sensor and pore. A reduction in the slope of the Boltzmann fit to the conductance-voltage curve in response to perturbations has also been suggested to reflect alterations in coupling (Yifrach, 2004). Although such functional effects are not unique to perturbations that disrupt coupling, these studies highlight the importance of various residues in the S4-S5 linker and the S6 tail regions during activation gating in voltage-gated ion channels.

Measurement of charge-voltage curves in addition to conductance-voltage curves can provide unique insight

with regards to the thermodynamics of this process (Chowdhury and Chanda, 2012). Mutants such as the ILT mutant (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999) in the Shaker potassium channel produce a distinct response whereby the conductance-voltage curve is shifted rightward but the charge-voltage curve is left shifted, implying that the mutation stabilizes the activated voltage sensor while at the same time destabilizing the open pore. Muroi et al. (2010) identified multiple positions in the S4-S5 linker and S6 region of the Na_v 1.4 channel, where a tryptophan substitution caused such opposite effects on voltage sensor and pore stability. Response function analysis of simple models showed that these contrasting shifts are produced when mutations modify interactions in both resting and activated conformations (Fig. 3). This is holds true for allosteric (Fig. 3 A, Scheme II) as well as obligatory models (Fig. 3 A, Scheme IV.) Mutagenesis studies in the Shaker potassium channel (Soler-Llavina et al., 2006; Haddad and Blunck, 2011) also identified additional sites that have opposite effects on voltage sensor and pore stability. It is important to note that the increased separation between the F-V (or Q-V) and G-V curves by itself cannot be used as an indicator of loss of coupling. Separation of the response curves is empirically related to conformational coupling but is not uniquely governed by it. As shown in Fig. 3, altering intrinsic stabilities of the individual structural units also increases the separation between the two response curves.

The high-resolution structures of voltage-gated ion channels (Long et al., 2005a, 2007; Payandeh et al., 2011) support the notion that this region involving the S4-S5 linker and S6 tail is important for electromechanical coupling. The voltage-sensing domain in the structure of the full-length channel forms limited contacts with the pore domain, except at the tight intracellular interface constituted by the S4-S5 linker, N-terminal end of S5 helix, and S6 tail. Although this contact is primarily intrasubunit, they are also seen to form intersubunit contacts (Long et al., 2007; Batulan et al., 2010).

In addition to the S4-S5 linker, another interface toward the external side has been implicated in electromechanical coupling (Long et al., 2007; Lee et al., 2009). This extracellular intersubunit gating interface between the S1 and S5 segments has strongly coevolved interactors and is also found in close proximity in the crystal structure. Perturbations at these interfaces exert strong effects on the gating process, and it has been proposed that the integrity of this interface is necessary for efficient force transmission from the S4 segment to the channel gates. Other studies have corroborated the functional importance of this interface (Bocksteins et al., 2011), although, at a mechanistic level, its role remains unclear.

As shown in Fig. 4, a comparison of sites implicated in electromechanical coupling shows that many of them are at the ends of the S4-S5 linker. This suggests that the flexible interhelical hinges may have a specific role. For efficient transfer of mechanical energy from the S4 to the S6 tail, S4-S5 linker and S4 need to move together as a rigid body. Such a rigid body motion will be aided when the angle between the S4 and S4-S5 linker is maintained as the channel activates, whereas the angle between the S4-S5 linker and S5 undergoes easy relaxations. The latter prevents the transfer of energy to the S5, which remains relatively unchanged in open and closed states. Therefore, we expect the hinge between the S4 and S4-S5 linker (the proximal hinge) to be relatively rigid (preventing large angle bending motions), whereas that between the S4-S5 liner and S5 (the distal hinge) is flexible (allowing relatively free-angle bending). Hinge flexing has been shown to be crucial for conformational coupling in several soluble proteins (Colonna-Cesari et al., 1986; Sharff et al., 1992; Kumar et al., 1999), for example in T4 lysozyme. Future experiments may shed more light on the role of amino acids at the ends of the S4-S5 linker.

Is the coupling interaction "attractive" or "repulsive"?

Coupling between the voltage sensor and the pore can be achieved in two very distinct ways: the force linking the two domains could be attractive in nature (pulling force) or repulsive (pushing force). According to the prevalent model of electromechanical coupling, the electrical force displaces the charged S4 segment, which pulls the covalently attached S4-S5 linker. The movement of the linker is transmitted to the S6 tail through the tight noncovalent interaction interface between them (Long et al., 2005b). This mechanism necessarily requires strong positive interactions to exist between the S4-S5 linker and S6 tail. Alternatively, one can envision that depolarization-induced movement of the voltage sensors relieves steric inhibition on the pore, which allows it to naturally open. In this scenario, the voltage sensors in the resting state exert a pushing force to keep the pore gates closed.

The two gating paradigms can be distinguished by measuring the innate state preference of the pore domain; i.e., the state (closed or open) that the pore domain "likes" to be in, in the absence of the voltage sensors. If the pore preferentially remains closed, electromechanical coupling would involve a pulling force, exerted by the activating voltage sensors on the channel gates. Conversely, if the pores like to stay open, the voltage sensors in their resting conformation apply a pushing force on the channel gates, keeping them closed. Upon depolarization, activation of the voltage sensors relieves this force, causing channel opening (Fig. 5). Consideration of the intrinsic stability of the open/closed state of the pore (and voltage sensors) is critical to understand the nature of forces driving electromechanical coupling.

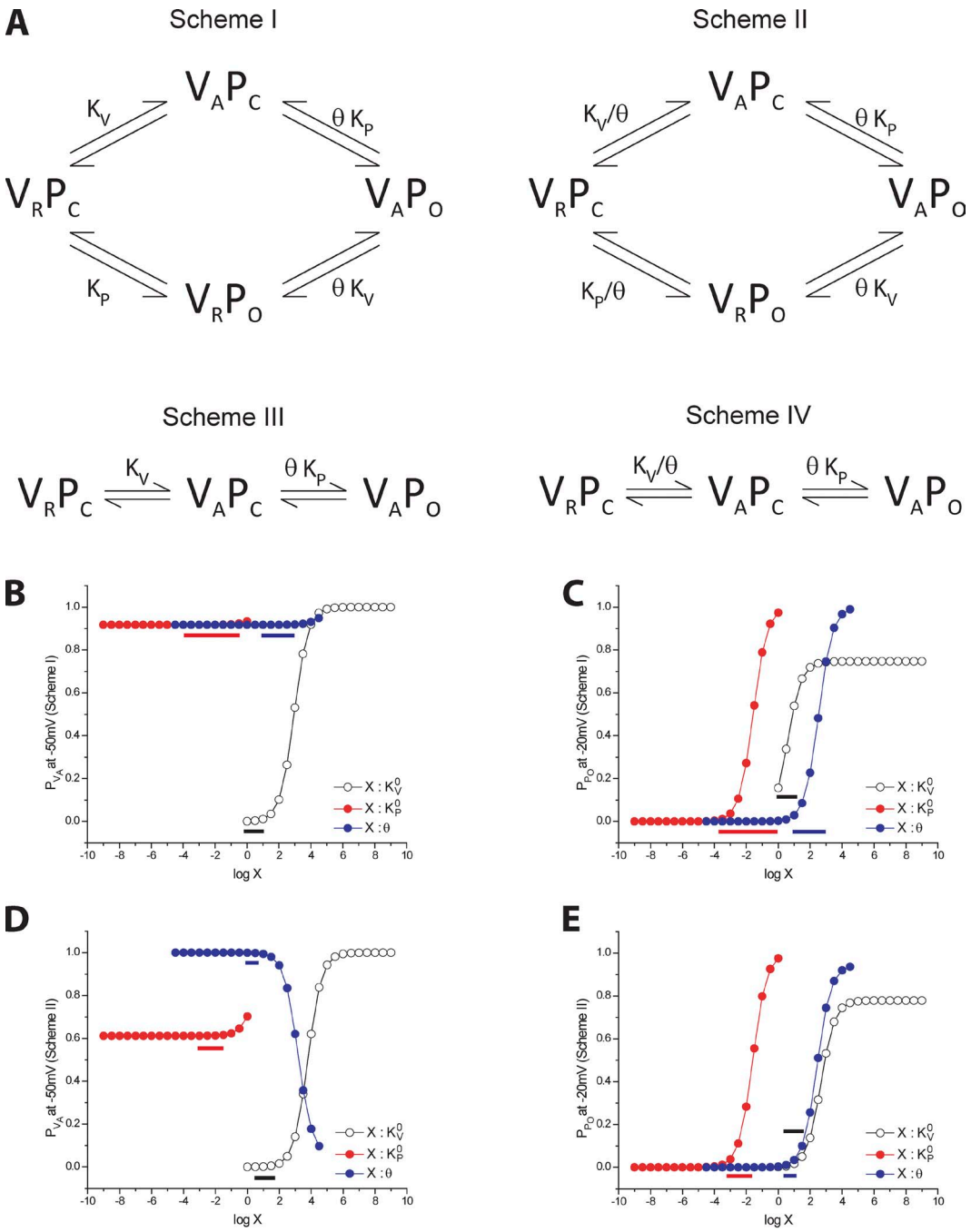

Figure 3. Coupling schemes to explain the opposite shifts in the activation of the voltage sensors and the pore. (A) Scheme I shows a canonical allosteric scheme showing voltage sensor activation (intrinsic equilibrium constant: K_V^0) and the pore opening (intrinsic equilibrium constant: K_P^0) being energetically connected via an interaction in the doubly activated state (where the voltage sensor is activated and the pore is open), represented by θ. Scheme II is a noncanonical counterpart of Scheme I, where the two structural units are interacting in both the doubly activated and the doubly resting state (i.e., the states where both the conformation are in "like" conformations). Schemes III and IV are obligatory analogues of Schemes I and II, respectively, where the conformational state with a resting voltage sensor and an open pore (V_RP_O) is eliminated. (B and C) The variation of occupancy of the activated state of the voltage sensor (P_{V_A} at −50 mV) and the open state of the pore (P_{P_O} at −20 mV) due to change in the different model parameters in Scheme I. The values for the simulations are taken from Muroi et al. (2010). Changing all the model parameters one at a time affects the two probability estimates similarly, which suggests that an opposite effect on the voltage sensor and the pore cannot be simply explained by Scheme I. The colored bars alongside the correspondingly colored curves depict the range of parameter values for which P_{V_A} is practically constant but P_{P_O} changes significantly. Such an effect would manifest in increased separation between the two curves. (D and E) The similar curves (as in B and C) obtained for Scheme II show that only when θ is changed do P_{V_A} and P_{P_O} change in an opposite way. For all other parameters, the two probability terms change in the same direction. The same observation will hold true for the obligatory schemes (III and IV); i.e., Scheme III will not yield a situation when the two probability measures are affected in opposite ways due to alteration of a single thermodynamic parameter, whereas in Scheme IV, the opposite shifts can be accomplished by altering θ.

Our initial insights regarding the innate stability of the pore came from high-resolution crystal structures of KcsA, a prokaryotic pH-gated potassium selective channel (Schrempf et al., 1995; Doyle et al., 1998; LeMasurier et al., 2001). These channels are tetrameric but comprise only two transmembrane segments per subunit. The structure showed a closed channel and revealed a hydrophobic bundle crossing at the intracellular end of S6. Functional studies suggested that the hydrophobic bundle crossing observed in KcsA is also present in the six transmembrane K_V channels and that strong hydrophobic interactions in these regions stabilize the closed state of the channel (Hackos et al., 2002; Yifrach and MacKinnon, 2002; Sukhareva et al., 2003; Kitaguchi et al., 2004). The implication of these studies is that the pore likes to stay closed, and upon depolarization, is pulled open by the voltage sensors. Nevertheless, direct measurements of the intrinsic stability of this domain by expressing the pore without the voltage-sensing domain suggest that the open pore might be more stable (Santos et al., 2008; Shaya et al., 2011). Potassium channels encoded by viruses, comprising only the S5-P-S6 segments with very short N and C termini, exhibit large open probability even at hyperpolarized voltages (Pagliuca et al., 2007). Thus, KcsA may be anomalous because it has a network of salt bridges and hydrogen bonds that stabilize the closed state (Thompson et al., 2008; Uysal et al., 2011). We should note that these observations were made in atypical ion channels and that, at present, it remains unclear to what extent these findings are applicable to the prototypical voltage-gated ion channels.

How does this alternate gating paradigm affect our interpretation of the mutations that alter electromechanical coupling? Of particular interest are those mutants that cause opposite shifts in the Q-V (F-V in case of sodium channels) and G-V curves (Ledwell and Aldrich, 1999; Soler-Llavina et al., 2006; Muroi et al., 2010; Haddad and Blunck, 2011). Mutants, which increase the separation between the Q-V and G-V curves along the voltage axis, have been interpreted to decrease coupling between the voltage sensor and pore on the basis of the assumption that the pore intrinsically prefers to be in a closed conformation. If, however, the isolated pore domain of Shaker or Na_v 1.4 prefers to be in an open conformation, then these mutants, which include the ILT mutant in the Shaker potassium channel, must increase coupling between the pore and voltage sensor. Although seemingly counterintuitive, it makes sense when one considers that the voltage sensors and pore are negatively coupled in this gating paradigm. Therefore, an increase in negative coupling would increase the energy difference between the activated voltage sensor and pore.

Other strategies to identify interaction networks

Molecular dynamics simulations and free-energy perturbation techniques are frequently used to computationally predict the strength of interaction between specific sites of the protein if a high-resolution structure is available. In some cases, the predicted interactions have been corroborated by functional measurements (Cordero-Morales et al., 2011). Nonetheless, as experimentalists, we are of the opinion that these energetic calculations have to be experimentally validated to assuage concerns regarding the sensitivity of the simulations to force fields and, thereby, the accuracy of calculated interaction energies.

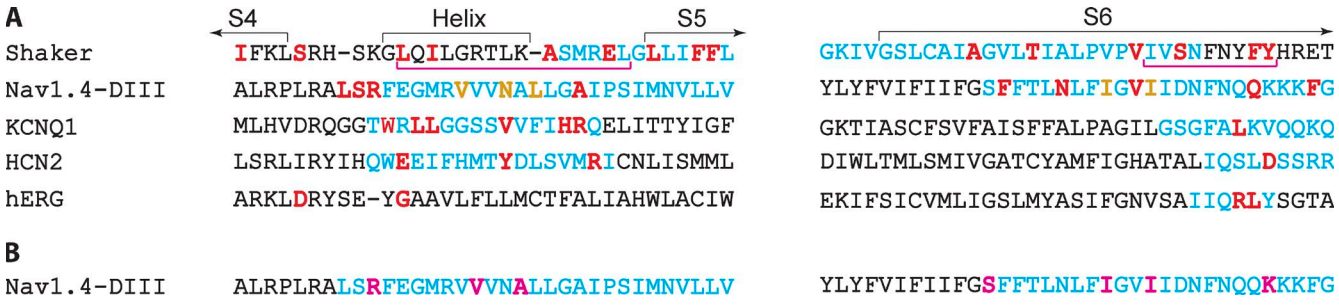

Figure 4. Important sites in the S4-S5 linker and S6 end region, which are putatively crucial for electromechanical coupling. (A) The S4-S5 linker and the S6 tail regions of five different voltage-gated ion channels are aligned. The demarcation of the helical regions is based on the structure of the K_V1.2/2.1 chimera. In blue are the regions of the protein that have been analyzed through scanning mutagenesis, and the residues marked in red are the ones which have been proposed to be involved in coupling voltage sensor and gate motions (*Shaker*: Ledwell and Aldrich, 1999; Soler-Llavina et al., 2006; Batulan et al., 2010, Haddad and Blunck, 2011; *Na_V1.4-Domain III*: Muroi et al., 2010; *KCNQ1*: Labro et al., 2008, 2011; *hERG*: Sanguinetti and Xu, 1999; Tristani-Firouzi et al., 2002, Van Slyke et al., 2010; *HCN2*: Chen et al., 2001; Decher et al., 2004). For the Shaker channel, the regions marked by the magenta bars are the complementary regions posited to be crucial for voltage sensor pore coupling in Lu et al. (2002). In the Na_V sequence, the brown residues are sites that might be involved in coupling but lack the distinct phenotypic response of opposite shifts in the FV and GV curves (unlike the positions in red). (B) An orthogonal assay of coupling performed in the Na_V1.4 channel (Domain III) using the lock-in strategy (see main text) identified sites (marked in magenta) within the same regions that are for the voltage sensor pore coupling (Arcisio-Miranda et al., 2010). Many of these residues coincide with or are close to sites that perturb coupling, as shown in Muroi et al. (2010; compare with residues marked in the Na_V sequence in A).

The relative interaction energies between a pair or specific network of residues can be calculated accurately using double mutant cycle analysis under certain conditions (Horovitz and Fersht, 1990; Yifrach and MacKinnon, 2002; Yifrach et al., 2009). In this method, the nonadditive component when both interactors are mutated defines the free energy of interaction. In particular, when a system exists in only two states, this relationship can be easily established. However, in a coupled system, the measured free energy term depends on multiple parameters, and unless it can be independently established that the mutations disrupt only pairwise interaction energy, mutant cycle analysis cannot be applied to estimate relative interaction energies. In the Shaker potassium channel, for instance, mutations at two distant sites in different subunits may produce an appearance of interaction in the G-V curves because the final pore transition is concerted. Also, as pointed out recently by Clapham and Miller (2011), mutations that convert a closed state to an open state in a multistate gating process will produce shifts in macroscopic conductance responses, a widely used measure of free energy of activation, although the underlying coupling energies may not change.

An alternate experimental approach that has been recently proposed to identify the network of interacting residues between two structural units is what we refer to as the "lock-in" strategy. In brief, the approach involves locking one structural unit in a "fixed" conformation and monitoring the energetics of the other structural unit. Locking one structural unit will have an effect on the free energy of activation of the other only when the two units are energetically coupled. Mutations that disrupt the interaction will attenuate the effect of locking one unit on the activity of the other. Such an approach was first applied to probe coupling in a voltage-gated sodium channel (Arcisio-Miranda et al., 2010). The pore was locked in a putatively open state through the application of a use-dependent channel blocker, lidocaine, whereas the voltage sensor was monitored through a site-specifically labeled fluorescent probe. The study identified multiple sites in the S4-S5 linker and S6 tail which, when perturbed (through a tryptophan substitution), result in a reduced effect of the pore lock-in on the movement of the voltage sensor (Fig. 4). The lock-in strategy identifies a limited set of state-dependent coupling interactions. For instance, in the lidocaine experiments, residues that are involved in interactions with the closed pore will not be identified. Thus, in this respect, χ^{diff} is a more comprehensive parameter to measure coupling interactions, although lock-in strategy is a useful orthogonal approach and has been used in other systems (Kohout et al., 2010; Ryu and Yellen, 2012).

Concluding remarks

Over the past decade, combined functional and mutagenesis studies have implicated the residues in the S4-S5 linker and the S6 end segments as the primary molecular determinants of electromechanical coupling. High-resolution structures of different voltage-gated ion channels derived from phylogenetically distant organisms show that the overall architecture and gating interfaces involved in voltage sensor and pore domain interaction is strongly conserved. However, important details such as the strength and state-dependence of these interactions remain obscure. This, for the most part, is due to our inability to obtain a direct estimate of the coupling interaction between different domains in an obligatorily coupled system, in contrast to allosteric systems. Although new structures, particularly of those in different states, will undoubtedly continue to be important, more effort will be required to develop new experimental and analytical tools to quantify these interactions and identify interactors. The importance of energetics is underscored by the fact that sometimes a single relatively conserved mutation can cause dramatic changes in the channel function, but only modest

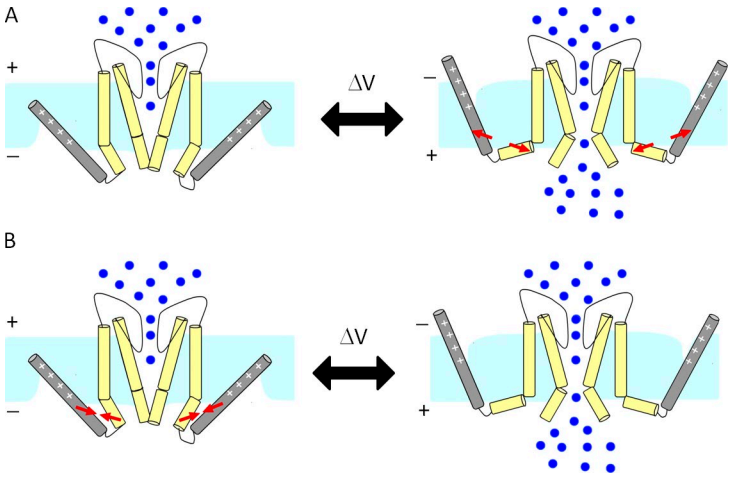

Figure 5. Forces governing voltage sensor pore coupling. (A) Voltage sensor pore coupling can be attractive. The voltage sensor in up conformation can pull the pore gates, which intrinsically tend to be closed, open. (B) The voltage sensor–pore interaction can be repulsive where the voltage sensor in resting state prevents the pore from opening. In this case, the pore intrinsically tends to be open, and repulsive forces between the two domains keep it closed when the voltage sensor is down conformation. The arrows show the direction of the forces. Note that these two forces are not necessarily exclusive because they occur in different conformations, and in theory they can both exist at the same time.

changes in crystallographically derived protein structures (Gonzalez-Gutierrez et al., 2012). Understanding the molecular driving forces behind protein conformation changes, in combination with high-resolution structures, will be the key for developing novel intervention strategies to treat ion channel pathophysiology.

This Perspectives series includes articles by Andersen, Colquhoun and Lape, and Horrigan.

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