Using hierarchical thermodynamic linkage analysis to study ion channel gating

Tzilhav Shem-Ad and Ofer Yifrach

Department of Life Sciences and the Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

The Journal of General Physiology

Ion channels are multi-subunit, multi-modal proteins that exhibit hierarchical organization, whereby the different components interact in a cooperative manner to realize a tunable function of the channel as a whole. Synergy is thus a fundamental aspect of ion channels threaded across all three complex system characteristics, namely, hierarchy, dynamism, and regulation. A full understanding of a system thus requires a formalism to assess the synergy between any two or more system components and across all system hierarchies. Thermodynamic linkage analysis (Wyman, 1964) offers such unified formalism, with the principle behind this approach being context dependence (Carter et al., 1984; Ackers and Smith, 1986). Two Perspectives addressing conformational coupling in voltage-gated ion channels (Chowdhury and Chanda, 2012) and in voltage- and ligand-activated BK channels (Horrigan, 2012) published in the December 2012 issue of the *JGP* highlighted the importance of domain-level linkage analysis for understanding how the coupling between gating and pore domains could lead to channel opening. Both papers demonstrated that when it comes to explaining the molecular details of such coupling, residue-level information, in particular for those residues found at domain interfaces, is required. Furthermore, knowing the magnitude and state dependence of residue interactions across such domain interfaces is essential for discriminating between possible gating mechanics scenarios. An accepted method for attaining such residue-level information is double mutant cycle coupling analysis (Horovitz and Ferst, 1990). Here, we point out that double mutant cycle analysis is, in fact, a context-dependent linkage analysis at a residue level and that, when properly applied, such linkage analysis is powerful and can reveal changes in residue interactions along the gating pathway of the channel. We further highlight that when combined, domain- and residue-level linkage analyses can reveal the molecular details of domain coupling leading to channel opening.

The conceptual analysis method of thermodynamic linkage (Wyman, 1964) offers a unified formalism for assessing interactions, as exemplified for the case of ligand-linked conformational changes found at the heart of the MWC classical allosteric model (Monod et al., 1965). In such formalism, the strength of nonadditive interactions and their contributions to protein function can be evaluated using the thermodynamic square presented in Fig. 1 A (Carter et al., 1984; Ackers and Smith, 1986), analogous, in essence, to Wyman's linkage cycle. In brief, at any level of channel protein (*P*) hierarchy, two components, *X* and *Y*, can be tested for coupling by comparing the magnitude of a change in one component in the absence or presence of a change in the second component, as revealed by comparing any two parallel transitions along the cycle.[1] If the free-energy change associated with a change in component *X* is independent of the change in the second *Y* component, i.e., $\Delta G_1 = \Delta G_2$, then there is no context dependence and the two components do not interact. If, however, a change in *X* depends on whether or not *Y* has already undergone a change, i.e., $\Delta G_1 \neq \Delta G_2$, then context dependence exists. In this case, the interaction free energy between the components is given by $\Delta G_1 - \Delta G_2$. The sign of this difference, i.e., "+" or "−" indicates whether the components interact in a positive or negative manner.[2] The linkage cycle in Fig. 1 A allows for the evaluation of the interaction energy between only the *X* and *Y* components, since, to first approximation, any interaction of these components with the rest of the protein cancels out when evaluating context-dependence changes, as reflected in the parallel transitions along the cycle ($\Delta G_1 - \Delta G_2$). The thermodynamic linkage analysis performed using the cycle presented in Fig. 1 A is general and can be applied to any level of system hierarchy to address the interplay between subunits, domains, or residues, cases in which we address synergy in quaternary, tertiary, and primary structures, respectively. Linkage analysis

[1]A change associated with a system component can relate to the activation of the component, either through a conformational transition or upon ligand binding, or mutation.

[2]In the case of a change involving a conformational transition, the sign of the coupling free energy dictates the state dependence, i.e., in what state the interaction is stronger.

Correspondence to Ofer Yifrach: ofery@bgu.ac.il

The Rockefeller University Press
J. Gen. Physiol. Vol. 141 No. 4 507–510
www.jgp.org/cgi/doi/10.1085/jgp.201310976

can be further applied at the level of protein modules, ignoring traditional borders of hierarchical structural levels as demonstrated, for example, for the case of two different ligand-binding sites within a single domain or for pore gates of ion channels.

In fact, past efforts have shown the generality of this approach. In the 1980s, Fersht and colleagues applied such linkage cycle at the residue level to evaluate the synergy between two interacting residues (Carter et al., 1984; Horovitz and Fersht, 1990). Although Fersht termed this approach "double mutant cycle analysis," it is, in essence, a context-dependent linkage analysis performed at the residue level. The use of linkage cycles to address synergy at the domain level was elegantly demonstrated for polymodal ion channels (Chowdhury and Chanda, 2013; Sigg, 2013), as further discussed in the Perspectives on conformational coupling alluded to earlier (Figs. 2 C and 3 A in Chowdhury and Chanda, 2012, and Horrigan, 2012, respectively). The same linkage cycle (Fig. 1 A) was further used at the quaternary structure level to address synergy among subunits during pore-opening transitions (Zandany et al., 2008) and at the module level to address the coupling between the lower activation and upper (slow) inactivation pore gates of potassium channels (Ben-Abu et al., 2009). This general pairwise linkage formalism can also be extended to higher dimensions for evaluating mutual coupling among any number of system components (Horovitz and Fersht, 1990), at all levels of hierarchy, as demonstrated when residue-level, high-order double mutant cycle linkage analysis was used to study *K*v channel gating (Sadovsky and Yifrach, 2007). Here, too, it is the context dependence of higher-order interactions that is being addressed (Horovitz and Fersht, 1990).

Now that the value of double mutant cycle coupling analysis in the broad context of thermodynamic linkage has been shown, we would like to comment on the ΔG values that are "allowed" in such analysis. Linkage analysis requires a thermodynamically measurable parameter associated with any structural or functional property of the protein that can reliably be measured and have its magnitude evaluated. This parameter must represent a discrete thermodynamic transition along the ligation or gating pathway of the protein;[3] it is not an overall thermodynamic parameter describing the output of the system as a whole. Residue-level double mutant linkage analysis is no different. Thus, as indicated by Chowdhury and Chanda (2012) in relation to voltage-dependent ion channel proteins, unless the channel is purely of the Boltzmann type, overall channel-opening free energies cannot be used in such analysis to evaluate the magnitude of pairwise interactions, as this system energy descriptor does not represent a discrete thermodynamic parameter.[4] Rather, many transitions in the gating pathway are lumped together in this overall parameter, where in each transition, coupling between the two residues being tested could differ.

Should the corollary of the above discussion discourage one from using double mutant cycle linkage analysis? The answer is no. In fact, when properly applied, double mutant cycle linkage analysis can provide insightful residue-level mechanistic information. To demonstrate this, consider the example of the obligatory channel gating scheme described in Fig. 1 B. In this scheme, there are two voltage-sensor transitions ($K_1(V)$ and $K_2(V)$), followed by a pore-opening transition (L). This scheme represents the core subunit mechanism of the *Shaker* voltage-dependent K^+ channel, as determined by the Aldrich and Sigworth groups (Zagotta et al., 1994; Schoppa and Sigworth, 1998). After detailed steady-state and transient kinetics analyses involving single- and many-channel recordings, the values of the chemical components of the equilibrium constants of all voltage-sensor and pore-opening transitions were determined for the wild-type channel. These values can also be determined for all "gating-intriguing" single and double mutants comprising a double mutant linkage cycle. If all single and double mutations do not change the channel gating pathway but instead only affect the equilibrium constants along the gating pathway (namely, $K_1(V)$ and $K_2(V)$ and L), then the approach is valid. When this information is available, following Horovitz et al. (1991), one can construct three sequential double mutant interaction cycles (Fig. 1 C) and use the appropriate K_1, K_2, and L chemical equilibrium constants to determine the relative interaction free energy between any two interesting residues in each of the channel states described in Fig. 1 B. Thus, changes in the coupling free energy between the two residues tested can be monitored along the gating pathway of the channel (Fig. 1 C), possibly yielding valuable residue-level information on the mechanics of channel gating.[5] In particular, such information can explain the opposing shifts in *Q-V* and *G-V* curves observed upon *K*v channel residue mutation, whether resulting from changes in voltage sensor–pore domain coupling or from changes in the intrinsic stability of the two domains.

[3]The equilibrium constants frequently used to calculate residue interactions in double mutant cycle linkage analysis are global thermodynamic reporters of the functional or structural changes associated with the wild-type or mutant proteins, e.g., ligand binding or conformational transition. A major advance in evaluating residue interactions using site-specific energetics, as determined by hydrogen exchange NMR measurements, was reported by Boyer et al. (2010).

[4]This statement is true even when accurately evaluating the total free energy of channel gating, using the median method analysis of $\bar{Y}$-*S* and *Q-V* curves of ligand-gated or voltage-gated channels (Chowdhury and Chanda, 2013), respectively.

[5]In principle, double mutant cycle analysis can also be applied using rate constants, yielding the interaction energies between the tested residues in the transition state. This adds another dimension to the analysis, namely, realizing the coupling free energies between residues along the reaction coordinate of channel gating.

Nonadditive contributions of two such residues in both *Q-V* and *G-V* curves hint at the involvement of the two residues in electromechanical coupling underlying channel opening. Analysis relying on a combination of sequential mutant interaction cycles (termed "COSMIC" analysis), as reflected in Fig. 1 C, has been applied previously to monitor changes in α-helical pairwise interactions along the trajectory of protein folding (Horovitz et al., 1991).

Finally, the added value of sequentially using domain-level and residue-level linkage analysis to better understand the mechanics of channel gating should be emphasized. The "holistic" domain-level thermodynamic linkage approach of Chowdhury and Chanda (2013) and Sigg (2013) allows for evaluation of interdomain couplings in nonobligatory allosteric systems in an essentially model-free manner and using reliable, traditional readouts (*Q-V*, *G-V*, and $\bar{y}$-*S* curves). Combined with scanning mutagenesis and structural information, one can use such domain-level linkage analysis to reliably detect those allosterically sensitive residues that mediate interdomain couplings, in particular those at gating–pore domain interfaces. These selected residues are targets for detailed steady-state and transient kinetics analysis aimed at revealing how (and by how much) the different equilibrium constants of all transitions along the channel gating pathway would be affected upon mutation. With this information in hand, interesting residue pairs can be functionally probed, using residue-level thermodynamic linkage analysis, to reveal changes in residue coupling along the channel gating pathway, as described above. Such information is important as it reveals not only the magnitude of coupling but also the state dependence of the interaction, thus helping us to better understand the molecular details of domain coupling.

To summarize, complex ion channel gating systems are highly hierarchical and exhibit shells of cooperative interactions of increasing magnitudes at all levels. It is

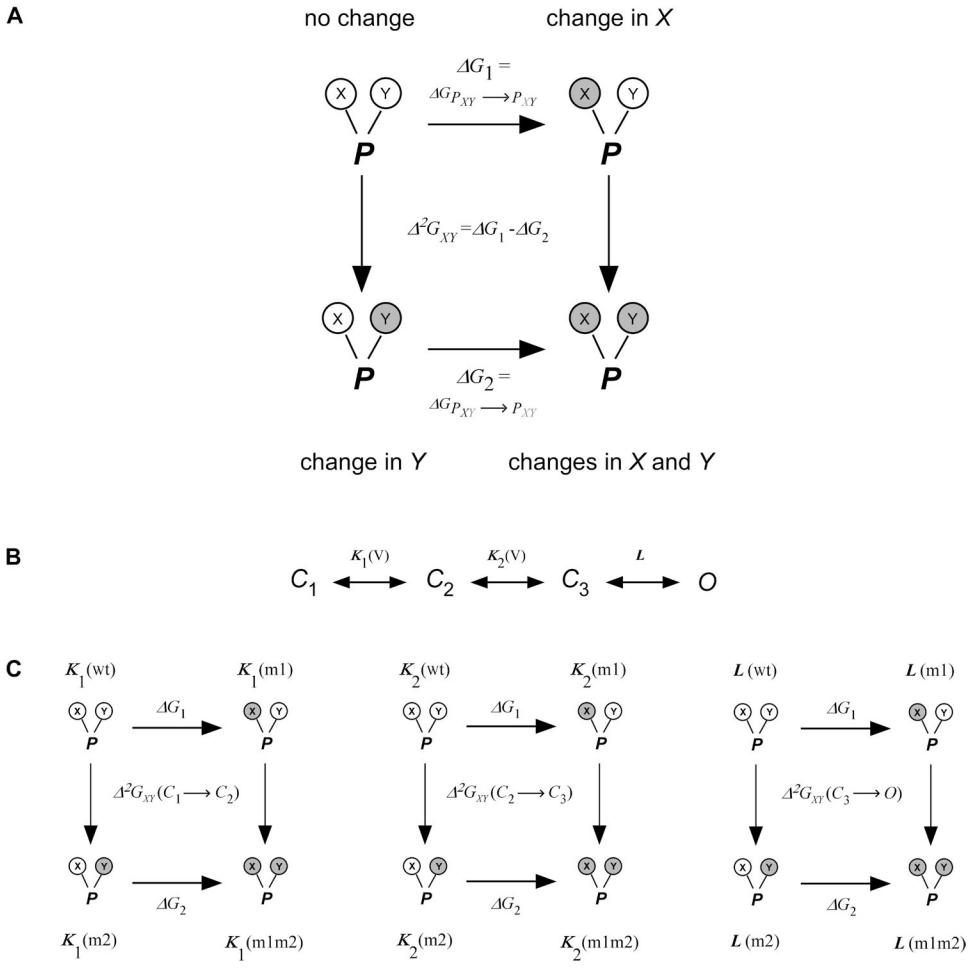

Figure 1. Hierarchical thermodynamic linkage analysis is used to study ion channel gating. (A) A unified thermodynamic linkage cycle for assessing the magnitude and sign of interaction between any *X* and *Y* protein (*P*) components. The cycle comprises four protein states, one in which no changes occurred in components *X* and *Y* (P_{XY}), two states in which one of the components has undergone a change (P_{Xy} and P_{xY}), and a fourth state in which changes occurred in both components (P_{xy}). ΔG_1 and ΔG_2 correspond to the free-energy changes for a change in component *X* to occur, in the absence and presence of a change in the second *Y* component, respectively. The nonadditive component of the two changes, as given by $\Delta G_1 - \Delta G_2$ ($=\Delta^2 G_{XY}$), determines the magnitude and sign of the interaction between the *X* and *Y* components. (B) An obligatory (subunit) channel gating scheme characteristic of the *Shaker* Kv channel. In this scheme, only after all four voltage sensors undergo two transitions ($K_1(V)$ and $K_2(V)$) does late concerted pore opening (L) occur. *C* and *O* denote the closed and open states, respectively. (C) Combination of sequential mutant interaction cycles to evaluate changes in the interaction free energy between the *XY* residue pair along the gating pathway described in B. In each cycle, the different wild-type (wt) and mutant (m) chemical equilibrium constants are indicated above the appropriate channel protein. In the left cycle, $\Delta^2 G_{XY}(C_1 \rightarrow C_2)$ denotes the interaction energy between the *XY* residue pair in the C_2 channel state relative to the C_1 state and is calculated according to $-RT\ln((K_1^{wt}\ K_1^{m1m2})/(K_1^{m1}\ K_1^{m2}))$. The other residue interaction energies are calculated in a similar manner. In all schemes, arrows represent equilibrium between the states connected.

thus only natural that the unified context-dependent thermodynamic linkage cycle of Wyman and Fersht be used to evaluate intersubunit, interdomain, inter-module, and inter-residue interactions, reflecting nonadditive functional effects at all levels of channel structural hierarchy. Integrating the information obtained will bring us closer to a coherent picture of molecular gating.

Work in the Yifrach laboratory is supported by the Israel Science Foundation (grant 488/12).

Olaf S. Andersen served as editor.

REFERENCES

Ackers, G.K., and F.R. Smith. 1986. Resolving pathways of functional coupling within protein assemblies by site-specific structural perturbation. *Biophys. J.* 49:155–165.

Ben-Abu, Y., Y. Zhou, N. Zilberberg, and O. Yifrach. 2009. Inverse coupling in leak and voltage-activated K^+ channel gates underlies distinct roles in electrical signaling. *Nat. Struct. Mol. Biol.* 16:71–79. http://dx.doi.org/10.1038/nsmb.1525

Boyer, J.A., C.J. Clay, K.S. Luce, M.H. Edgell, and A.L. Lee. 2010. Detection of native-state nonadditivity in double mutant cycles via hydrogen exchange. *J. Am. Chem. Soc.* 132:8010–8019. http://dx.doi.org/10.1021/ja1003922

Carter, P.J., G. Winter, A.J. Wilkinson, and A.R. Fersht. 1984. The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell.* 38:835–840. http://dx.doi.org/10.1016/0092-8674(84)90278-2

Chowdhury, S., and B. Chanda. 2012. Perspectives on: Conformational coupling in ion channels: Thermodynamics of electromechanical coupling in voltage-gated ion channels. *J. Gen. Physiol.* 140:613–623. http://dx.doi.org/10.1085/jgp.201210840

Chowdhury, S., and B. Chanda. 2013. Free-energy relationships in ion channels activated by voltage and ligand. *J. Gen. Physiol.* 141:11–28. http://dx.doi.org/10.1085/jgp.201210860

Horovitz, A., and A.R. Fersht. 1990. Strategy for analysing the co-operativity of intramolecular interactions in peptides and proteins. *J. Mol. Biol.* 214:613–617. http://dx.doi.org/10.1016/0022-2836(90)90275-Q

Horovitz, A., L. Serrano, and A.R. Fersht. 1991. COSMIC analysis of the major α-helix of barnase during folding. *J. Mol. Biol.* 219:5–9. http://dx.doi.org/10.1016/0022-2836(91)90852-W

Horrigan, F.T. 2012. Perspectives on: Conformational coupling in ion channels: Conformational coupling in BK potassium channels. *J. Gen. Physiol.* 140:625–634. http://dx.doi.org/10.1085/jgp.201210849

Monod, J., J. Wyman, and J.-P. Changeux. 1965. On the nature of allosteric transitions: A plausible model. *J. Mol. Biol.* 12:88–118.

Sadovsky, E., and O. Yifrach. 2007. Principles underlying energetic coupling along an allosteric communication trajectory of a voltage-activated K^+ channel. *Proc. Natl. Acad. Sci. USA.* 104:19813–19818. http://dx.doi.org/10.1073/pnas.0708120104

Schoppa, N.E., and F.J. Sigworth. 1998. Activation of *Shaker* potassium channels. III. An activation gating model for wild-type and V2 mutant channels. *J. Gen. Physiol.* 111:313–342. http://dx.doi.org/10.1085/jgp.111.2.313

Sigg, D. 2013. A linkage analysis toolkit for studying allosteric networks in ion channels. *J. Gen. Physiol.* 141:29–60. http://dx.doi.org/10.1085/jgp.201210859

Wyman, J., Jr. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein Chem.* 19:223–286. http://dx.doi.org/10.1016/S0065-3233(08)60190-4

Zagotta, W.N., T. Hoshi, and R.W. Aldrich. 1994. *Shaker* potassium channel gating. III: Evaluation of kinetic models for activation. *J. Gen. Physiol.* 103:321–362. http://dx.doi.org/10.1085/jgp.103.2.321

Zandany, N., M. Ovadia, I. Orr, and O. Yifrach. 2008. Direct analysis of cooperativity in multisubunit allosteric proteins. *Proc. Natl. Acad. Sci. USA.* 105:11697–11702. http://dx.doi.org/10.1073/pnas.0804104105