

## CFTR gating: Invisible transitions made visible

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By tracking conformational transitions of individual ion channel proteins, single-channel recordings allow the observer to deduce gating mechanisms, as well as estimate rate constants of microscopic gating steps. For most ion channels, gating mechanisms are complex, comprising multiple stable open (O) and closed (C) conformations among which conformational transitions may follow a network of possible kinetic pathways. The ensemble of all states and the connectivities among them are called a gating scheme. The strictest limitation to deciphering complex gating schemes is the fact that only transitions between closed and open states can be detected, whereas transitions among closed states, or among open states, remain invisible in single-channel current traces. It is true that such silent transitions are betrayed by the shapes of the dwell-time distributions, which carry information both on the numbers of closed and open states and on the magnitudes of the rate constants of “invisible” transitions (Colquhoun and Hawkes, 1981). But in practice, extracting such information is not at all straightforward: whereas fitting a mechanism to the data by maximum likelihood is a convenient way for estimating rate constants once the underlying gating scheme is known, deciphering the mechanism itself is a much harder task. Typically, several alternative schemes provide satisfactory fits, and comparison of log-likelihood scores facilitates the choice among models only under specific circumstances (when one model is a subset of the other). Thus, clearly, rendering an invisible transition as directly observable in a single-channel current trace is extremely informative for addressing mechanisms. In the previous issue of *The Journal of General Physiology*, Zhang and Hwang exploit mutants in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel that allow visualization of a normally invisible gating transition. In doing so, they reveal a role for the internal vestibule of the channel in gating.

For the majority of ion channels, gating is an equilibrium process that does not require an external source of energy. An equilibrium implies that the frequency of transitions along each microscopic kinetic pathway is identical in both directions, so that no preferential

cycling may occur around any kinetic loop. One consequence of such microscopic reversibility is that all observable features of a single-channel current recording must be time reversible: any time asymmetry signals violation of thermodynamic equilibrium and hence suggests an external source of energy input (Rothberg and Magleby, 2001). A second consequence of microscopic reversibility is that the dwell-time distributions must decay monotonically: a peaked distribution is again indicative of a nonequilibrium process (Kijima and Kijima, 1987).

CFTR, the chloride channel mutated in cystic fibrosis patients, is the only ion channel member of the large family of ABC transport proteins. A large body of information confirms that opening and closing (gating) of its ion pore follows the same conserved mechanism that drives thermodynamically uphill substrate transport in most ABC proteins. In these transporters, a cycle of ATP binding and hydrolysis at two cytosolic nucleotide-binding domains (NBD1 and 2) is linked to transmembrane domain (TMD) movements that alternately expose the substrate binding site to the two sides of the membrane. The CFTR protein has also been shown to hydrolyze ATP and this activity found necessary for its channel function (Li et al., 1996). That finding raised the possibility that CFTR gating might be coupled to its enzymatic activity. Given that the free energy change ( $\Delta G$ ) of ATP hydrolysis is highly negative under physiological conditions (and even more so under *in vitro* conditions in the nominal absence of ADP and inorganic phosphate), any such coupling would imply a cyclic gating mechanism far from thermodynamic equilibrium.

Several lines of experimental evidence have confirmed the aforementioned hypothesis by demonstrating violation of microscopic reversibility for CFTR gating. In heavily filtered (10 Hz) single-channel recordings of WT CFTR reconstituted in lipid bilayers, the presence of the organic buffer MOPS in the cytosolic compartment caused the appearance of a unique subconductance pattern: channels preferentially opened to a lower conductance level (termed O<sub>1</sub>) but briefly

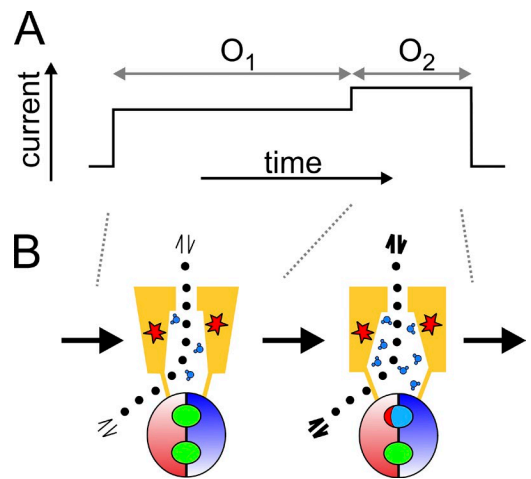
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transitioned to a higher conductance level (termed  $O_2$ ) before closing (Fig. 1 A). Furthermore, mutations that disrupt ATP hydrolysis in CFTR and other ABC proteins eliminate this obvious time asymmetry (Gunderson and Kopito, 1995). This subconductance pattern, observed at low bandwidth, corresponds to a differential pattern of rapid flickery block by the deprotonated form of the blocker (MOPS<sup>-</sup>) and reflects a change in the binding affinity of the blocker at some point within each open-channel event (Ishihara and Welsh, 1997). Based on the observation that the  $O_1 \rightarrow O_2$  conductance transition is not observed when ATP hydrolysis is prevented, it seemed natural to suggest that the subconductance transition coincides with ATP hydrolysis. Thus, the  $O_1$  and  $O_2$  conductance states were ascribed to pre- and post-hydrolytic states of the NBDs, respectively (Fig. 1 B).

Further support for a nonequilibrium gating cycle was provided by the shapes of CFTR's dwell-time distributions: both the distribution of closed (interburst) dwell times (when observed at subsaturating ATP concentrations; Zeltwanger et al., 1999) and open (burst) dwell times (Csanády et al., 2010) were found to display peaks. In the latter study, a mutation that disrupts ATP hydrolysis was shown to convert the open-time distribution to a monotonically decaying distribution. These peaked distributions for WT CFTR were again consistent with a cyclic mechanism in which channels normally shuttle through (at least) two sequential closed states (ATP free  $\rightarrow$  ATP bound) before opening and then through (at least) two sequential open states (pre-hydrolytic  $\rightarrow$  post-hydrolytic; Fig. 1 B) before closing.

Much structural and functional work on CFTR and related ABC proteins has outlined many of the mechanistic details of this unique gating cycle. In the presence of ATP, NBDs form head-to-tail dimers with two ATP molecules occluded in interfacial binding sites. Such ATP-bound dimers are extremely stable but dissociate after ATP hydrolysis (Moody et al., 2002). In a subset of asymmetric ABC proteins, including CFTR, only one composite binding site is catalytically active (the "canonical site"), whereas the other "degenerate" site is inactive, so that each cycle of NBD dimerization/disruption includes only one ATP hydrolysis event (Fig. 1 B; Basso et al., 2003). This NBD catalytic cycle is coupled to TMD conformational changes. In ABC exporters, tight NBD dimerization flips the TMDs from inward to outward facing, and dimer disruption resets them to inward facing (Hollenstein et al., 2007). On the contrary, dimer formation in CFTR opens the pore and dimer disruption closes it (Vergani et al., 2005). Thus, WT CFTR open (burst) events have been proposed to involve occlusion of a single ATP molecule at the canonical site and to be terminated predominantly by hydrolysis of that ATP (Vergani et al., 2005; Csanády et al., 2010), consistent with the observations of Gunderson



**Figure 1. Time-asymmetric conductance pattern of CFTR correlates with its ATP hydrolytic activity.** (A) Illustration of the  $O_1$ - $O_2$  phenotype. Schematic representation of single-channel current for a typical single open (burst) event, illustrating the two subconductance levels in their preferential order. (B) Cartoon representation of pre- (left) and posthydrolytic (right) open states of a CFTR channel. Arrows between the two states reflect the preferential order in which they are visited in each burst, as a consequence of a nonequilibrium gating cycle. TMDs, yellow; NBD1, red; NBD2, blue; ATP, green; ADP + phosphate, blue oval + red crescent; chloride ions, black spheres; water molecules, blue. The canonical ATP-binding site is depicted as the top site in the NBDs, and the noncanonical site is shown at the bottom. Red stars in the TMDs represent mutations that disturb the local charge distribution. In the posthydrolytic state, a suggested expansion of the internal vestibule results in the accommodation of a larger number of water molecules, which dampen the negative impact of the mutations on chloride throughput rate (stronger double arrows bracketing the chain of chloride ions).

and Kopito (1995). However, later work proposed the possibility of more than one ATP occlusion event within a single open burst (Jih et al., 2012b), a hypothesis supported by the appearance of multiple  $O_1 \rightarrow O_2$  subconductance transitions within a single burst of a CFTR pore mutant (Jih et al., 2012a). Clearly, more work will be needed to fully understand the details of this unique gating cycle, and with such open questions at hand, studying the mechanism underlying the time-asymmetric changes in the conductance properties of CFTR has become increasingly important.

Despite numerous studies that have clarified the molecular mechanism of anion selectivity and conductance properties and outlined the rough structural organization of the open CFTR pore (Linsdell, 2017), no clues have emerged that would explain the  $O_1$ - $O_2$  subconductance pattern caused by MOPS<sup>-</sup> or similarly by 3-nitrobenzoate (Csanády and Töröcsik, 2014) until recently. The first hint about a region that might be involved in this phenomenon came from work in the Hwang laboratory (Jih et al., 2012a): neutralization of the positive charge of a pore-lining arginine, located in the intracel-

lular vestibule at position 352 of TM helix 6 (TM6), caused the appearance of the  $O_1$ - $O_2$  subconductance pattern even in the absence of MOPS<sup>-</sup>. Given that MOPS<sup>-</sup> is a large organic anion that blocks by binding to the intracellular vestibule, this finding suggested that the region in the vicinity of R352 might be involved in the same open-pore conformational change that also affects MOPS<sup>-</sup> binding affinity.

In their new, elegant study, Zhang and Hwang (2017) provide the first coherent set of information regarding the structural underpinnings of this interesting phenomenon. They show that the local arrangement of surface charges in the vicinity of position 352 of the internal vestibule indeed plays a crucial role in determining the conductance pattern of a burst. Whereas the local charge distribution found in WT CFTR ensures that the permeation rate for chloride ions remains identical throughout an open burst, specific perturbations of this “native” charge distribution (Fig. 1 B, red stars) preferentially reduce chloride conduction rate in the  $O_1$  state, causing the appearance of an  $O_1$ - $O_2$  subconductance pattern. The authors identify several different ways of disturbing the native charge distribution—neutralization of the natively positive charge at TM6 position 352 (R352C and R352Q) or introduction of a negative charge into natively neutral TM5 position 306 (N306D and N306E; these two positions are located at similar depths in the internal vestibule, at the spot where the latter tapers down to a narrow tunnel [Zhang and Chen, 2016])—all of which lead to the same result. But it is not simply the magnitude of the total charge that matters because the authors show that transplanting charges to different positions, without affecting total charge, can also cause the appearance of the  $O_1$ - $O_2$  phenotype (e.g., R352Q/M348R, R303C/S310R, and R303C/F311R). Clearly, both the magnitude and the precise location of the charges are important. Furthermore, it is rewarding to see that the effects on conductance pattern of the aforementioned mutations and of MOPS<sup>-</sup> are not independent of each other. Whereas some of the mutations further amplify the inherent difference in MOPS<sup>-</sup> affinities of the  $O_1$  and  $O_2$  states (F311C), others abolish this difference, eliminating the ability of MOPS<sup>-</sup> to elicit the  $O_1$ - $O_2$  phenotype (e.g., R303C).

What is the mechanism behind the differential chloride conduction rates of the  $O_1$  and  $O_2$  states in the mutants? One effect that a positive surface charge in the vestibule of an anion channel may exert is to increase the local concentration of chloride ions, and the net positive surface charge in CFTR's internal vestibule clearly does play such a role. CFTR chloride conductance is a hyperbolic function of chloride concentration, and in WT channels, conductance is half-maximal at ~50 mM chloride (Linsdell et al., 1997). In contrast, for the N306D

mutant (in which net positive surface charge is reduced), conductance is shown to be smaller than that of WT in low (~50 mM) but to approach that of WT at very large (~1 M) chloride concentrations. The conductance-concentration curve of the mutant, consistent with a  $K_{0.5}$  of ~300 mM, indeed reports a reduced apparent affinity of the pore for permeating chloride, but this is shown to apply identically to the  $O_1$  and the  $O_2$  state. Moreover, whereas a change in local chloride concentration in the internal vestibule would be expected to preferentially affect inward currents at negative potentials (reflecting outward chloride ion flow), the authors show that the  $O_1$ - $O_2$  conductance pattern of the N306D mutant is voltage independent and can be observed even for outward currents recorded at positive membrane potentials. It thus seems unlikely that the increase in chloride conductance between states  $O_1$  and  $O_2$  of this (and other) mutants would reflect an increase in the local chloride concentration in the internal vestibule. Instead, the  $O_1$ → $O_2$  conformational change of the mutated vestibules must affect the maximal chloride throughput rate, which depends on the rates of dehydration/binding and unbinding of permeating anions in the pore. The authors suggest an expansion of the internal vestibule, which accompanies the  $O_1$ → $O_2$  transition, as a possible explanation (Fig. 1 B): mutation-induced changes in the electrostatic potential that a permeating chloride ion experiences in the  $O_1$  state might be dampened in the  $O_2$  state by a larger number of water molecules in the vestibule (Fig. 1 B). This could explain why mutations preferentially reduce conductance in the  $O_1$  state relative to the  $O_2$  state, causing the appearance of the  $O_1$ - $O_2$  conductance pattern.

Beyond the novel insights provided, a further merit of this study by Zhang and Hwang (2017) is that it has generated a valuable toolbox in the form of mutants that allow visualization of the  $O_1$ → $O_2$  transition. These tools open up new pathways for further studies aimed at a better understanding of both the process of chloride ion permeation through the open pore and of the ATP-dependent gating cycle. Along that first line, it will be important to establish whether and how the anion permeability sequence changes between the  $O_1$  and  $O_2$  states to decipher how the  $O_1$ → $O_2$  transition affects the energetics of anion-protein interactions. Along the second line, it will be important to understand exactly what molecular rearrangements take place at the NBDs concurrent with the TMD rearrangements that cause the (now observable!)  $O_1$ → $O_2$  transition. Last but not least, all of these future studies will benefit tremendously from high-resolution structures of CFTR, an abundant source of information which has finally become more than just a dream (Zhang and Chen, 2016).

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