

Degenerate ABC composite site is stably glued together by trapped ATP

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An article published in this issue (see Tsai et al.) provides important advances in our understanding of the gating mechanism of the CFTR, the chloride channel mutated in cystic fibrosis patients. Indeed, the relevance of the conclusions goes beyond understanding CFTR, extending at least to the subfamily of ABC proteins to which CFTR belongs, and perhaps also to the entire superfamily.

ABC proteins are found in all organisms from bacteria to human and mediate transmembrane export/import of a variety of substrates at the expense of ATP hydrolysis. The 48 human ABC proteins are mostly exporters involved in a wide variety of physiological processes, ranging from insulin secretion to antigen presentation, cholesterol and bile salt transport, and drug detoxification (Dean and Annilo, 2005). Despite the evolutionary divergence reflected by the heterogeneity of transported substrates, the basic architecture and transport mechanism of these proteins are highly conserved. The core structure of all ABC proteins is built from two homologous halves, each consisting of a transmembrane domain (TMD) and a cytosolic nucleotide binding domain (NBD). The TMDs form the pathway for uphill substrate translocation, energized by an ATP hydrolysis cycle catalyzed by the two NBDs. A vast body of biochemical studies compiled over two decades, fertilized by recently obtained high-resolution crystal structures, has clarified the outlines of this process at a molecular level. At the heart of this functional cycle lies the formation of an intramolecular dimer by a head-to-tail association of the two NBDs. Dimerization is induced by interfacial binding of two ATP molecules that act as molecular glue; in the glued-together dimer, the ATPs are bound in composite sites formed between the “head” of one NBD and the “tail” of the other. Both the head and the tail side contain conserved sequence motifs important for ATP binding and hydrolysis, such as the Walker A and B motifs (head) and the ABC signature motif (tail). In crystal structures of full-length ABC exporters, obtained in various conformations, a nucleotide-bound NBD dimer is always associated with an outward-facing TMD conformation, whereas separated monomeric NBDs (observed in structures devoid of ATP) go along

with an inward-facing TMD conformation (Hollenstein et al., 2007; Procko et al., 2009). Thus, flipping between outward- and inward-facing TMD conformations, a fundamental property of uphill transporters (Jardetzky, 1966), is coupled to formation/dissociation of the NBD dimer. Because the dimer is very stable with two ATPs at the interface, dynamic cycling requires ATP hydrolysis to allow prompt dimer disassembly. An important question under intense debate is whether NBD separation is a large-scale movement that disengages the entire dimer interface, or whether the two composite ATP binding sites open up one at a time to maintain a partial dimer conformation that continues to occlude one ATP.

In most ABC proteins, the two NBDs are either identical or highly conserved, allowing ATP hydrolysis to occur at both composite sites. Two models have been proposed for the catalytic cycle of such symmetrical ABC proteins. The “processive clamp” model (Janas et al., 2003) suggests that disruption of the NBD dimer requires hydrolysis of both ATP molecules, entailing complete separation of the interface to allow ADP-ATP exchange. In contrast, the “alternating catalysis” model (Senior et al., 1995) postulates that the two sites adopt alternating roles in consecutive cycles, such that in each cycle only one of the ATPs is hydrolyzed, followed by partial disengagement of the dimer sufficient to allow nucleotide exchange at only that site.

In a subset of ABC proteins, however, the two NBD sequences are highly divergent. The C subfamily of human ABC proteins, which includes the sulphonylurea receptors (SUR1 and SUR2) and the multidrug resistance proteins, as well as CFTR, belongs to this subset. In these asymmetric ABC proteins, non-conservative substitutions adjacent to the Walker B motif of the N-terminal NBD (NBD1) and in the signature sequence of the C-terminal NBD (NBD2) render the composite binding site formed by these motifs (composite site 1) catalytically inactive (Procko et al., 2009). Moreover, photolabeling experiments using 8-N₃-ATP suggest that, whereas ATP is turned over at a rate on the order of one per second at the active composite site 2 (formed by the

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NBD2 head and NBD1 tail), ATP remains stably bound to the NBD1 head for up to several minutes without being hydrolyzed (Szabó et al., 1999; Ueda et al., 1999; Gao et al., 2000; Aleksandrov et al., 2002; Basso et al., 2003). The intriguing question is whether this “occluded” ATP must be trapped between the NBD1 head and NBD2 tail of a formed composite site 1—as might be expected for a cycle that alternates between a full NBD dimer and a partial dimer—or whether ATP occlusion is an intrinsic ability of the NBD1 head by itself; the latter would leave room for the possibility of complete dimer separation in each catalytic cycle.

In their present article, Tsai et al. (2010) convincingly answer this question for the model ABC-C family member, CFTR. CFTR is unique among ABC proteins in that its TMDs form a pore that is permeable to chloride ions (Riordan et al., 1989). From an evolutionary perspective, CFTR is a broken ABC transporter in which the inward-facing TMD conformation represents the closed, and the outward facing conformation represents the open, chloride permeation pathway. On the other hand, gating of the CFTR chloride channel uses the fundamental mechanisms shared by all asymmetric ABC proteins driven by formation/dissociation of the NBD1/NBD2 heterodimer. Using the exceptional power of real-time recording of ion channel gating transitions, the authors tackle the fundamental question of whether the NBD dimer fully dissociates in each gating cycle; and they provide strong evidence that it does not.

The present work builds on tools developed over the past five years. Previous work by the same group has identified and characterized *N*⁶-(2-phenylethyl)-ATP (P-ATP) as an ATP analogue that opens CFTR channels with ~50-fold higher affinity ($K_{1/2}$ of ~1.6 μ M) than ATP. Open probability is also higher in saturating P-ATP than in saturating ATP due to both faster channel opening and slower closing (Zhou et al., 2005). Because in the continued presence of P-ATP both composite sites likely become loaded with this analogue, an obvious question to ask is whether the latter two effects on gating can be associated with P-ATP occupancy of one or the other composite binding site. Two lines of evidence suggested that prolongation of open times is due to binding of the analogue to composite site 1. First, the effect on the open times has a higher affinity than the effect on the closed times (Zhou et al., 2005), and biochemical experiments have identified composite site 1 as the higher-affinity site. A second hint was provided by the effect of P-ATP on “locked-open” events. Blocking hydrolysis at composite site 2 is known to prolong the lifetime of open events by approximately two orders of magnitude. This can be achieved either by mutating catalytically important residues in the NBD2 head, or by applying mixtures of ATP and a nonhydrolyzable ATP analogue like AMP-PNP or pyrophosphate (PP_i) (Gunderson and Kopito, 1994; Hwang et al., 1994;

Carson et al., 1995); in the latter case, the long locked-open events are due to formation of an NBD dimer in which ATP is bound in composite site 1, whereas AMP-PNP or PP_i is bound in composite site 2 (Tsai et al., 2009). In the presence of P-ATP, not only were normal open times prolonged, but so were the durations of locked-open events—whether induced by NBD2 head mutations (Zhou et al., 2005) or by coapplication of AMP-PNP (Zhou et al., 2005) or PP_i (Tsai et al., 2009).

Having characterized channel gating under conditions in which both composite sites are loaded with the same type of nucleotide (i.e., either ATP or P-ATP), in the present study Tsai et al. (2010) designed a clever ligand exchange protocol to study the gating properties of single CFTR channels with a different nucleotide bound at each of the two composite sites. Their idea is based on the very different nucleotide release rates of the two sites suggested by the biochemical experiments. Exploiting this anticipated difference, the authors monitor the pattern of gating of single CFTR channels intermittently exposed to either ATP or P-ATP, and extract mean open and shut times over consecutive short time windows. They find that the pattern of gating changes in two clearly separable steps in response to a rapid ligand exchange. Upon replacing ATP with P-ATP, they find that the opening rate increases instantaneously, whereas the prolongation of open times follows only with an ~30-s delay. In the interim, the channel performs 20–30 gating cycles with unchanged open time. Similarly, when they replace P-ATP with ATP, the opening rate decreases instantaneously, whereas the prolonged open times characteristic of P-ATP persist for another 30–50 s. During this interval, the channel produces some dozens of long openings before reverting to the briefer openings characteristic of ATP. This two-step adaptation is also reflected by the time course of macroscopic current increase upon an ATP→P-ATP switch, which is bi-exponential, with a rapid (<1 s) and a slow (~50 s) time constant. Moreover, when they remove P-ATP after a brief exposure, they obtain a fast current decay like that upon the removal of ATP, whereas the removal of P-ATP after a prolonged exposure is followed by a slower current decay signaling prolonged channel open times. So, clearly, while opening rate is an instantaneous reflection of the type of nucleotide present in the bath, channel closing rate has a long-term “memory” dissipated over a time course orders of magnitude longer than the cycle time.

The obvious interpretation is that this memory is provided by the nucleotide occluded in the inactive composite site 1. If so, then the time course of the slow kinetic change is a measure of the speed at which this site is vacated, confirming that nucleotide remains bound here for many gating cycles. To test whether this is indeed the case, Tsai et al. (2010) remove an aromatic side chain in the NBD1 head, which in crystal structures

of NBD1 stacks against the adenine base of ATP. As anticipated, this perturbation dramatically speeds memory dissipation; the two-step character of kinetic changes upon ligand exchange is no longer resolvable in single-channel recordings, and, accordingly, the slow time constant of the macroscopic response is shortened to ~ 2 s. Thus, when nucleotide binding at the NBD1 head is destabilized, channel closed and open times both change almost instantaneously upon ligand exchange.

The stage is now set to pose the main question: Does nucleotide occlusion at site 1 require structural contributions from the NBD2 tail? To answer this question, the authors introduce mutations into the NBD2 signature sequence—the part of the NBD2 tail that completes composite site 1 in a formed NBD1-NBD2 dimer. The clear-cut result is that several of these NBD2 tail mutations dramatically speed memory dissipation, just the same way as the removal of the conserved aromatic side chain in the NBD1 head. Thus, the nucleotide that is stably occluded at site 1 for several gating cycles clearly interacts with both the NBD1 head and the NBD2 tail. This is an elegant and irrefutable demonstration that during a typical gating cycle, the NBD dimer interface remains closed around composite site 1, and opens up only partially at the hydrolytically active composite site 2, to allow ADP-ATP exchange there. This lack of movement at composite site 1 provides a satisfying explanation for the generally small effects on gating of perturbations at this site (Powe et al., 2002; Csanády et al., 2005; Zhou et al., 2006; Muallem et al., 2008), and is an important revelation of the catalytic mechanism of all asymmetric ABC proteins.

As a further step, the authors study the rare instances of separation of composite site 1. Clearly, this must occasionally occur to allow ligand exchange there. By analyzing state dependence of the speed of ligand exchange at site 1, they show that this ligand is exchangeable only while the channel is shut, but not while it is open. The implication is that in a closed channel, in which the NBD2 head and the NBD1 tail are already separated, this separation will occasionally spread to composite site 1 and result in full separation of the dimer. However, such full separation is a rare event, and the authors provide evidence that once this happens, reformation of an occluded site 1 might require prior closure of composite site 2.

Questions remain. The inferred 30–50-s time constant for nucleotide exchange at site 1 falls short of explaining at least 20-fold longer lifetimes of 8- N_3 -ATP occlusion at NBD1 suggested by biochemical experiments (Aleksandrov et al., 2002; Basso et al., 2003). And the clear requirement of the NBD2 tail for this occlusion seems difficult to reconcile with apparently unaltered retention of 8- N_3 -ATP by NBD1 of a truncated CFTR construct lacking NBD2 (Aleksandrov et al., 2008).

What might be the reason for this discrepancy? Certainly, the presence of the azido group required for labeling cannot be the culprit, because Tsai et al. (2010) show that 8- N_3 -ATP occluded at site 1 is exchanged at a similar rate as ATP. Potential pitfalls of the biochemical experiments are limited time resolution and the difficulty of quantifying the efficiency of labeling. Because these signals are normalized to the apparent “0 time” signal, it is conceivable that they might miss the most relevant part of a time course that has a 30-s time constant, and instead report on a small fraction of channels in which, for some reason, occlusion persists for longer time periods. In any case, future studies will be needed to identify the functional relevance of such longer-term nucleotide binding and the structural correlate of the corresponding occluded state.

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