

Review

ATP hydrolysis-driven gating in cystic fibrosis transmembrane conductance regulator

Daniella Muallem and Paola Vergani*

Neuroscience, Physiology and Pharmacology, Faculty of Life Sciences, University College London, Gower Street, London WC1E 6BT, UK

Proteins belonging to the ATP-binding cassette superfamily couple ATP binding and hydrolysis at conserved nucleotide-binding domains (NBDs) to diverse cellular functions. Most superfamily members are transporters, while cystic fibrosis transmembrane conductance regulator (CFTR), alone, is an ion channel. Despite this functional difference, recent results have suggested that CFTR shares a common molecular mechanism with other members. ATP binds to partial binding sites on the surface of the two NBDs, which then associate to form a NBD dimer, with complete composite catalytic sites now buried at the interface. ATP hydrolysis and γ -phosphate dissociation, with the loss of molecular contacts linking the two sides of the composite site, trigger dimer dissociation. The conformational signals generated by NBD dimer formation and dissociation are transmitted to the transmembrane domains where, in transporters, they drive the cycle of conformational changes that translocate the substrate across the membrane; in CFTR, they result in opening and closing (gating) of the ion-permeation pathway.

Keywords: CFTR; ion channel; ATP-binding cassette transporter; ATP binding and hydrolysis; gating

1. INTRODUCTION

Cystic fibrosis is one of the most common lethal autosomal recessive disorders in Caucasian populations, affecting approximately 60 000 people worldwide (Guggino & Stanton 2006). The molecular basis of the disease was identified in 1989 (Riordan *et al.* 1989): patients had a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), which resulted in abnormal electrolyte permeability in various exocrine epithelia (Quinton 2007). The sequence clearly identified CFTR as a member of the superfamily of ATP-binding cassette (ABC) proteins (Dean & Annilo 2005; Hollenstein *et al.* 2007a). Most of these are integral transmembrane proteins that harness the chemical potential of ATP to transport very diverse compounds (referred to as allocrites) across membranes, often against steep electrochemical potential gradients. However, purified reconstituted CFTR was shown to act as a channel (Bear *et al.* 1992), providing a permeation pathway for anions to cross membranes, driven by their electrochemical potential difference. Recent research has suggested that the CFTR channel employs the molecular mechanism of its transporter relatives in its own individual way: ATPase cycles do not power uphill allocrite transport but simply the opening and closing

of the channel's gates. In this paper, we will review the evidence supporting this view.

2. STRUCTURE AND MECHANISM OF ABC TRANSPORTERS

The minimal functional unit of ABC transporters is thought to comprise two transmembrane domains (TMDs), which line the path across the membrane for transported allocrites, and two nucleotide binding domains (NBDs), which bind and hydrolyse ATP (Hollenstein *et al.* 2007a). In most eukaryotic transporters, as in CFTR, these four domains are encoded by a single gene comprising an N-terminal TMD1 and NBD1 and a C-terminal TMD2 and NBD2.

The numerous NBD structures solved to date reveal a conserved fold (Hung *et al.* 1998; Hopfner *et al.* 2000; Gaudet & Wiley 2001; Karpowich *et al.* 2001; Yuan *et al.* 2001; Locher *et al.* 2002; Smith *et al.* 2002; Chen *et al.* 2003; Schmitt *et al.* 2003; Verdon *et al.* 2003a,b; Lewis *et al.* 2004; Thibodeau *et al.* 2005; Zaitseva *et al.* 2005, 2006; Dawson & Locher 2006, 2007; Procko *et al.* 2006; Ramaen *et al.* 2006; Hollenstein *et al.* 2007b; Oldham *et al.* 2007; Pinkett *et al.* 2007; Ward *et al.* 2007), comprising a RecA-like subdomain ('head'), and a helical subdomain, unique to ABC transporters ('tail'). In the head subdomain, conserved motifs include the Walker A and B motifs involved in nucleotide coordination and hydrolysis, while the conserved ABC signature sequence (LSGGQXXR) is in the tail subdomain.

* Author for correspondence (p.vergani@ucl.ac.uk).

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NBDs can assume a dimeric ‘head-to-tail’ arrangement. In ATP-bound crystals, NBDs form tight dimers so that two composite ATP-binding sites are formed at the interface, each ATP molecule contacting the conserved motifs from the head of one NBD and the signature sequence from the tail of the partner (Smith *et al.* 2002; Chen *et al.* 2003; Zaitseva *et al.* 2005; Dawson & Locher 2007; Oldham *et al.* 2007; Ward *et al.* 2007). At the NBD–NBD interface, the two ATP molecules mediate a large part of the intersubunit interactions (acting as ‘molecular glue’, Karpowich *et al.* 2001), and indeed biochemical experiments carried out with isolated prokaryotic NBDs confirmed the importance of the ATP molecules, and in particular of the γ -phosphates, in stabilizing the dimeric conformation (Hopfner *et al.* 2000; Moody *et al.* 2002; Horn *et al.* 2003; Janas *et al.* 2003; Verdon *et al.* 2003a). These observations led to the hypothesis that an ATPase cycle involving formation and dissociation of tight NBD dimers (ATP binding \rightarrow tight NBD dimerization \rightarrow hydrolysis \rightarrow loss of the γ -phosphate \rightarrow opening of the NBD–NBD interface) could drive the cyclical conformational changes in the TMDs resulting in uphill allocrite transport (Hopfner *et al.* 2000).

High resolution structures of several full length ABC transporters, including both importers and exporters, have been solved recently (Dawson & Locher 2006, 2007; Hollenstein *et al.* 2007b; Oldham *et al.* 2007; Pinkett *et al.* 2007; Ward *et al.* 2007). The overall architecture of the TMDs is not conserved and in some the central cavity is accessible from the cytoplasm (inward facing) and in others from the extracellular space (outward facing). However, in all the crystals, most of the NBD/TMD contacts are seen to occur via a pair of structurally conserved α -helices, the ‘coupling’ helices. It has been suggested that the conformational signal generated by the binding of ATP and NBD dimerization could be transmitted to the TMDs via the movement of the coupling helices closer to each other, leading to a flipping of the TMD conformation from inward facing to outward facing (Hollenstein *et al.* 2007a).

3. ATP BINDING TO CFTR'S NBDS PRECEDES CHANNEL OPENING

Recent results have suggested that a similar cycle of ATP binding, NBD dimerization and ATP hydrolysis also plays an important part in controlling CFTR channel gating. In addition, CFTR requires phosphorylation by cAMP-dependent protein kinase (PKA) (Tabcharani *et al.* 1991; Csanády *et al.* 2000; Hegedus *et al.* 2008). The target serines are within the regulatory or ‘R’ domain, which is not present in other ABC transporters. Once the channels are phosphorylated, interaction with ATP controls the channel gates (figure 1). The molecular mechanism underlying the regulation of gating by ATP in pre-phosphorylated channels will be our focus here, since its structural underpinnings are common in CFTR and transporters.

As with bacterial ABC transporters, the N-terminal NBD1 and the C-terminal NBD2 of CFTR are thought to associate to form an intramolecular NBD1/NBD2

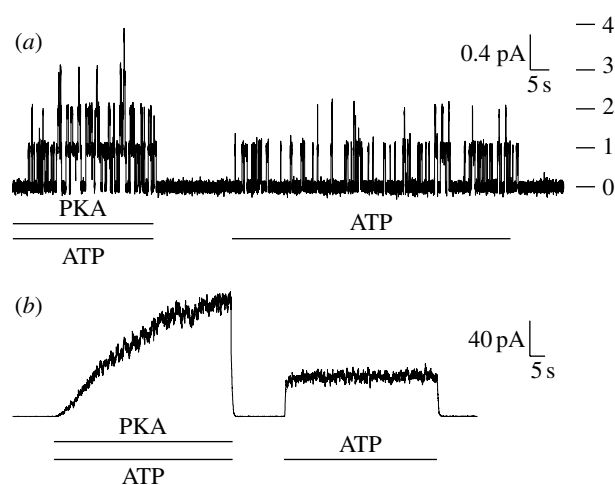


Figure 1. CFTR gating is regulated by PKA-mediated phosphorylation and by interaction with ATP. Current records from excised, inside-out patches, from *Xenopus* oocytes expressing WT CFTR. (a) Patch containing a small number of channels. Transitions between the closed and the open state of individual channels can be detected. On the right, horizontal lines show current levels reached by the indicated number of simultaneously open channels. Bars beneath traces indicate changes in solution composition on the cytosolic side of the patch. (b) Patch containing hundreds of WT channels. Pre-phosphorylated channels can be opened by exposure to ATP alone. After ATP removal, all channels close within seconds.

‘heterodimer’ with two composite binding sites formed at the interface (Kidd *et al.* 2004; Vergani *et al.* 2005; Mense *et al.* 2006). We here refer to the composite site comprising NBD1 Walker motifs and NBD2 signature as site 1, while site 2 will comprise NBD2 Walker motifs and NBD1 signature. In CFTR, as in several other eukaryotic ABC transporters (all the other members of the human ABCC subfamily, MRP1-9, SUR1 and SUR2 (Dean *et al.* 2001), as well as TAP1/TAP2 (Procko *et al.* 2006)), only interfacial site 2 presents conserved, consensus residues in all the motifs involved in ATP binding and hydrolysis. By contrast, on both faces of site 1 there are non-conservative substitutions at key residues. These result in the degenerate site 1 binding tightly, but not efficiently hydrolysing the bound ATP (Aleksandrov *et al.* 2001, 2002; Basso *et al.* 2003). When CFTR is reacted with the photoactive nucleotide 8-azido ATP, labelled on the γ -phosphate (8-azido [γ 32 P] ATP), radioactivity is retained at site 1, even after prolonged (30 min) incubation at 30°C (Cui *et al.* 2006). If, after incubation with 8-azido [α 32 P] ATP, CFTR is washed extensively at 30°C in nucleotide-free solution before photocrosslinking, nucleotide is seen to dissociate from site 1 with a time constant of approximately 15 min (Basso *et al.* 2003).

The [ATP] dependence of single-channel kinetic parameters was investigated to determine at which stage of the gating cycle ATP binding is required (figure 2). Reducing [ATP] does not affect average open burst duration. However, at low [ATP], the closed dwell time, the average time spent in the closed state between openings, is increased (Gunderson & Kopito 1994; Venglarik *et al.* 1994; Winter *et al.*

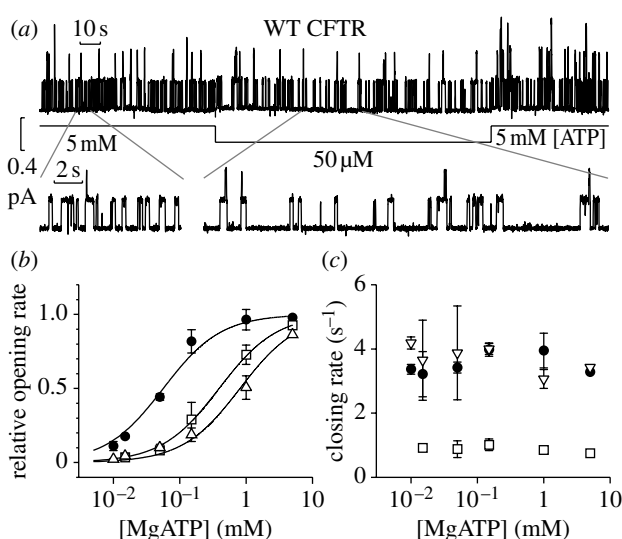


Figure 2. Rate of channel opening is [ATP] dependent. (a) Sample WT trace. Segments with expanded time scale are shown below. (b) Hyperbolic relationship between [ATP] and opening rates (apparent dissociation constants are 56 ± 5 , 807 ± 185 , $391 \pm 118 \mu\text{M}$ for WT (filled circles), K464A (open triangles) and D1370N (open squares), respectively). (c) Closing rates are [ATP] independent. Modified from Vergani *et al.* (2003).

1994; Zeltwanger *et al.* 1999; Csanády *et al.* 2000; Vergani *et al.* 2003). For wild-type (WT), a simple hyperbolic equation with an apparent dissociation constant of approximately $50 \mu\text{M}$ ATP describes the dose–response curve of opening rate as a function of [ATP] (figure 2b, filled circles). Thus, at sub-saturating [ATP], a binding step appears to rate-limit channel opening, indicating that ATP binding occurs on closed channels and is required for channel opening.

To determine whether one or both composite sites in the NBD1/NBD2 dimer are involved in channel opening, we introduced mutations at residues belonging to the conserved Walker motifs seen to interact directly with the bound nucleotide in solved crystal structures. For both mutants (K464A in site 1 and D1370N in site 2), opening rate was reduced at low [ATP], but fast opening could be restored by increasing [ATP] (figure 2b, open symbols). These results led us to conclude that binding at either sites can be made rate limiting for channel opening, suggesting that only after ATP binds to both head subdomains does the transition to the open state become energetically favourable (Vergani *et al.* 2003). However, Zhou *et al.* (2006), using a similar experimental strategy, obtained very different results. Sites seen to interact with the adenine moiety of ATP were mutated. The dose–response curve of channel opening rate as a function of [ATP] was seen to shift dramatically to the right when the consensus site 2 was mutated (Y1219G or Y1219I) but not when the degenerate site 1 was altered (W401G). These authors conclude that ATP binding at site 2 initiates channel opening (Chen & Hwang 2008). It is likely that the rate of opening for channels with no ATP bound at site 1 (monoligated channels) can be altered by mutations at the NBD interface around site 1. Possibly W401G channels open frequently as monoligated,

while WT, with its high-affinity site 1, only rarely would. More research is needed to clarify the role played by ATP binding at site 1 in channel gating.

In any case, ATP binding—at one or both NBDs—occurs on closed channels and is required for channel opening. In the light of recent advances in our structural understanding of ABC transporters, this electrophysiological and biochemical evidence is consistent with the hypothesis that after ATP binding, formation of a tight NBD1/NBD2 dimer is coupled to conformational changes in the TMDs opening the diffusion pathway for anions.

4. HYDROLYSIS AT THE CONSENSUS SITE 2 ALLOWS FAST CHANNEL CLOSURE

Closing rate of WT CFTR and mutants is not strongly affected by [ATP] (figure 2c, open symbols, Gunderson & Kopito 1994; Venglarik *et al.* 1994; Winter *et al.* 1994; Csanády *et al.* 2000; Vergani *et al.* 2003, but cf. Zeltwanger *et al.* 1999). However, CFTR, like other ABC proteins, is an active ATPase (Li *et al.* 1996), and blocking the hydrolytic cycle, through addition of either inorganic vanadate (Baukowitz *et al.* 1994; Gunderson & Kopito 1994) or non-hydrolysable nucleotide analogues (Gunderson & Kopito 1994; Hwang *et al.* 1994), slows channel closing (figure 3). The effect of such compounds can be mimicked by introducing mutations at key catalytic positions on both sides of composite site 2: in the head of NBD2 (Carson *et al.* 1995; Gunderson & Kopito 1995; Ramjeesingh *et al.* 1999; Zeltwanger *et al.* 1999; Vergani *et al.* 2003) and in the tail of NBD1 (Vergani *et al.* 2005). Equivalent mutations at the corresponding residues in site 1 (which in CFTR has lost catalytic activity, see §3) do not significantly affect closing rate (Carson & Welsh 1995; Gunderson & Kopito 1995; Ramjeesingh *et al.* 1999; Powe *et al.* 2002; Vergani *et al.* 2003), except in particular non-hydrolytic conditions (Powe *et al.* 2002; Vergani *et al.* 2003). These experiments suggest that hydrolysis of the nucleotide bound at site 2 triggers the rapid channel closure seen in WT (figures 1 and 3a: fast current decay after removal of ATP in control conditions).

Again, the evidence is consistent with the idea of NBD dimerization controlling CFTR's gates: fast channel closing would follow dissociation of the tight NBD dimer caused by hydrolysis of the nucleotide bound at site 2. The equilibrium between the closed and open conformations for the channel complex with two bound ATP molecules would be shifted towards the open channel conformation, so that closing through reversal of the opening transition (non-hydrolytic pathway) would occur rarely. For the majority of openings, hydrolysis, triggering release of the γ -phosphate and weakening of inter-NBD contacts would provide an alternative rapid pathway for channel closure.

Minor changes in closing rate have been reported caused by manipulations not expected to alter the hydrolytic rate. For example, the W401G mutant, with a weakened contact with the adenine of the ATP molecule bound at site 1, presented a less than twofold reduction in mean open time, interpreted by the

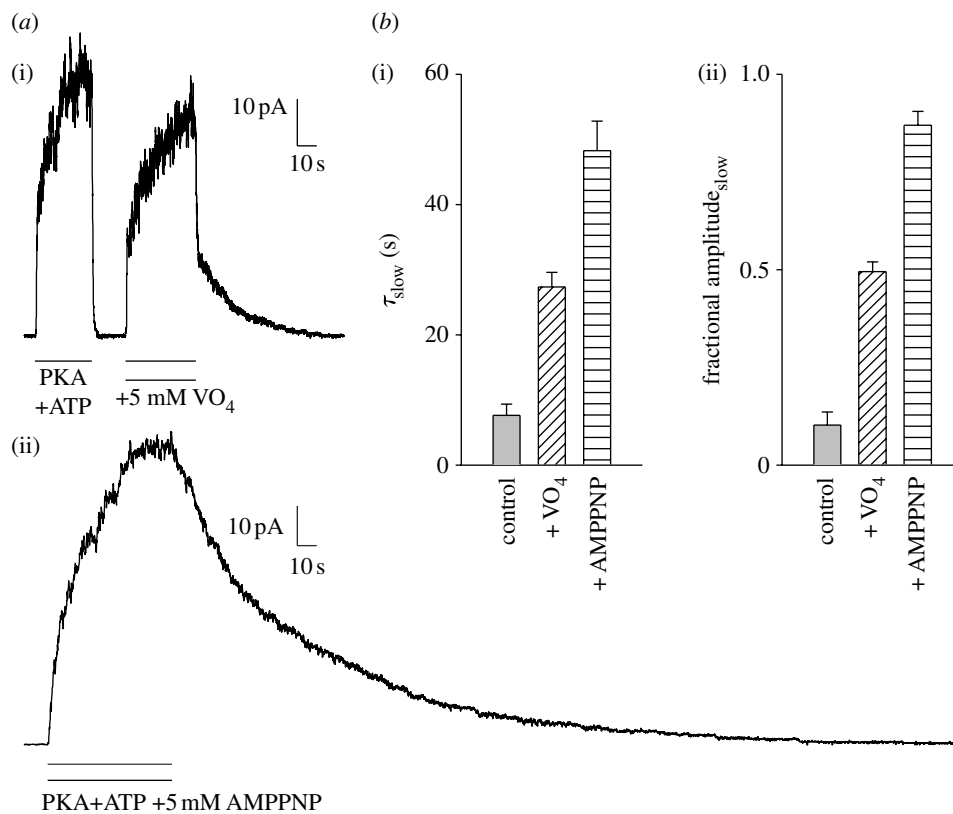


Figure 3. Blocking the hydrolytic cycle slows channel closing. (a) Because opening rate falls to virtually zero after ATP removal, time course of current decay measures channel closing rate. (i) Time constant for current decay in control conditions is less than 1 s, but a slow component ($\tau_{slow} = 21$ s) appears if opening occurs in the presence of orthovanadate (VO₄). In other ABC transporters VO₄ inhibits ATPase activity by acting as an analogue of the pentacovalent transition state intermediate of hydrolysis. (ii) Adenosine 5'-(β,γ -imido)triphosphate (AMPPNP) can also 'lock' channels open. (b, c) Mean (\pm s.e.m., $n \geq 15$) (i) time constant (τ_{slow}) and (ii) fractional amplitude of the slow component.

authors as suggesting that separation of the NBD dimer at site 1 can constitute the rate-limiting step in channel closing (Zhou *et al.* 2006). It is likely that the destabilization of the NBD dimer caused by a weaker interaction with ATP at site 1 would speed up dimer dissociation, and therefore closure, following ATP hydrolysis at site 2.

5. CFTR OPENING AND TIGHT NBD DIMERIZATION

Can we link the opening of the CFTR gate to the formation of an NBD1/NBD2 dimer more directly? To do this, we studied the energetic coupling between two residues on opposite sides of the consensus site 2, before, during and after channel opening.

R555 is in the signature sequence in the NBD1 tail; T1246 is in the Walker motif in the NBD2 head. The corresponding residues are seen to form a hydrogen bond in ATP bound, dimeric NBD crystals (Smith *et al.* 2002; Chen *et al.* 2003; Zaitseva *et al.* 2005, 2006). In addition, multiple sequence analysis (Lockless & Ranganathan 1999) on a sequence alignment comprising more than 10 000 NBD sequences (<http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00005>) indicated that this pair of positions had co-evolved. Changes in side chain at one position had been compensated by changes at the other, so that most existing sequences contain two side chains capable of forming a hydrogen bond that positions

the two α -carbons at an approximately 10 Å distance: either an arginine–serine (or an equivalent arginine–threonine, as in WT CFTR), or a lysine–asparagine pair (see figure 4b).

To study energetic coupling we applied mutant cycle analysis (Serrano *et al.* 1990; Fersht 1999). The WT, the two single mutants (R555K and T1246N) and the double mutant (R555K T1246N) form the corners of a thermodynamic cycle (figure 4a). If the two residues are independent, then the effects of mutations in WT and in mutant background (on parallel sides of the mutant cycle) will be the same. Any non-zero difference between mutation-linked changes on parallel sides of the cycle signifies—and can be used to quantify—energetic coupling between the two target residues ($\Delta\Delta_{int}$ in figure 4a; see also supplementary notes to Vergani *et al.* 2005).

We used mutation-linked changes in activation free energy for the opening reaction to follow energetic coupling between the two target residues as the channel opens. Both single mutations slowed channel opening, but in the double mutant fast opening was partially restored (figure 4c). The effects on parallel sides of the mutant cycle are therefore very different, yielding a significant energetic coupling ($\Delta\Delta G^{\ddagger}_{int}(\text{opening}) = -2.7 \pm 0.5$ kT), consistent with the formation of a stabilizing interaction in the transition state for the opening reaction that was not present in the closed ground state.

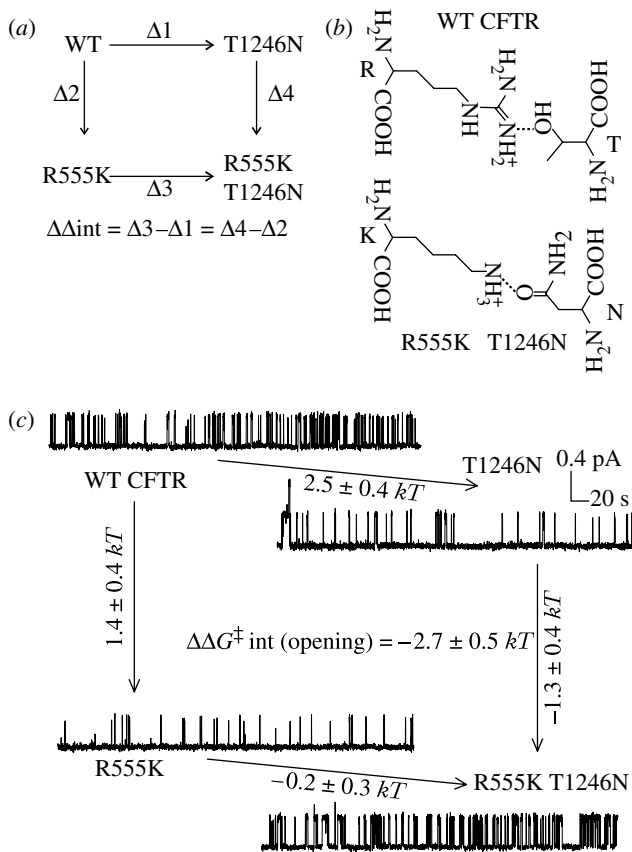


Figure 4. R555 and T1246 become coupled as the channel opens. (a) Schematic of the thermodynamic mutant cycle. ‘ Δ ’ beside arrows represents a mutation-linked change in any path-independent measurement. (b) Representation of hydrogen-bonded arginine–threonine and lysine–asparagine pairs. (c) Mutant cycle using changes in activation free energy ($\Delta\Delta G^{\ddagger}$) for the opening reaction reveal that R555 and T1246 become energetically coupled as channels approach the open state. For each mutation introduced (arrow) the mutation-linked change in ΔG^{\ddagger} (mean \pm s.d.) is indicated. Modified from Vergani *et al.* (2005).

We next used open probability (P_o) measurements in non-hydrolytic conditions to infer how energetic coupling changes as the channel gates between closed and open states. For channels containing a mutation impairing hydrolysis, the gating scheme is reduced to a simple closed-to-open equilibrium at saturating [ATP]. In these conditions, measurements of channel P_o can be used to calculate the free energy difference between the closed and open states. Introducing the T1246N mutation in a non-hydrolytic background (mutated at a crucial lysine, K1250R; Lerner-Marmarosh *et al.* 1999) strongly destabilized the open state with respect to the closed one. However, the same T to N mutation, when introduced in the R555K K1250R non-hydrolytic background, did not significantly alter the closed-to-open equilibrium. The sign and magnitude of the calculated coupling energy ($\Delta\Delta G_{int}(\text{open-closed}) = -2.4 \pm 1.0 kT$) are consistent with the two residues forming a stabilizing interaction, most likely a hydrogen bond, in the open state that is absent in the ATP-bound closed state (Vergani *et al.* 2005).

To examine coupling in the closed state preceding opening, we analysed the energetics of ATP binding.

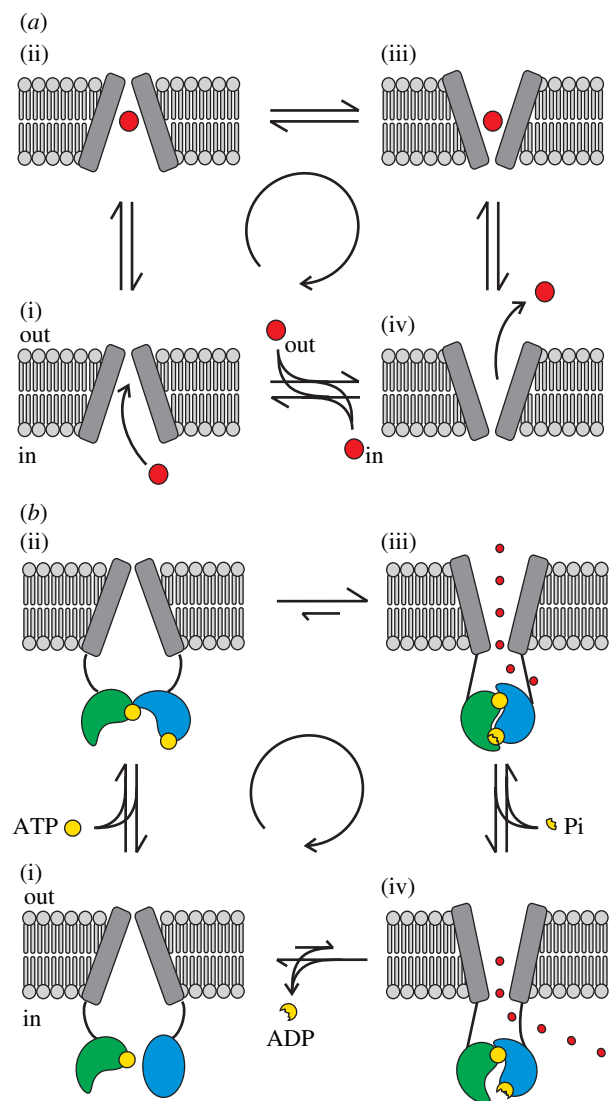


Figure 5. Conformational changes occurring in the TMDs of an exporter and in CFTR. (a(i–iv)) Cartoon representing an exporter actively transporting an allocrite (large red circle), through an alternating access and release mechanism. After a sufficient period of time the transporter will have cycled around the four conformations resulting in the net transport of allocrite. The coupled free energy releasing process is not shown. (b(i–iv)) Schematic of ATPase-driven CFTR gating. After ATP (yellow circle) binding, formation of a tight NBD1(green)/NBD2(blue) dimer is coupled to a conformational change in the TMDs, that opens the (external) channel gate. Although each step is reversible, ATP hydrolysis drives cyclic conformational changes resulting in a time asymmetry in single channel current records (Gunderson & Kopito 1995; Hennager *et al.* 2001). The number of anions (small red circles) crossing the membrane during each cycle is not stoichiometrically related to the number of ATP molecules hydrolysed: throughout the time the gate is open, the rate of anion flow will depend on the electrochemical potential gradient. However, on average, more than 10^6 Cl^- ions cross the membrane during one CFTR gating cycle (i.e. more than 10^6 ions/ATP), far more than the 1 (or half) allocrite per ATP molecule moved by the transporter relatives.

The apparent dissociation constant obtained from [ATP] dependence of opening rate is, for CFTR (Vergani *et al.* 2005), a reasonable estimate for the real dissociation constant for the ATP binding reaction

at site 2. The R555K mutation did not significantly affect apparent affinity, while the T1246N mutation reduced it to the same degree whether the residue at position 555 was WT R or mutant K. The effects of the T to N mutation in WT and mutant background are thus similar, yielding a negligible energetic coupling between the two target residues ($\Delta\Delta G_{\text{int}}(\text{unbound-bound}) = 0.3 \pm 0.5 \text{ kT}$). This indicates that any coupling is similar in the closed states before and after ATP binding. Because in all solved ATP bound structures the residue corresponding to the T1246 acceptor also acts as a donor in a hydrogen bond to a γ -phosphate oxygen, it is highly unlikely that binding of ATP would not alter the interdomain hydrogen bond involving T1246. It is more likely that there is no significant energetic coupling between T1246 and R555 in either closed state, neither before nor after ATP binding, with the NBD arrangement more similar to the nucleotide free dimeric crystals (Locher *et al.* 2002; Chen *et al.* 2003; Hollenstein *et al.* 2007b; Pinkett *et al.* 2007).

In summary, we have followed the interaction between two sites on opposite sides of CFTR's composite site 2: the target residues are not coupled in closed states that bind ATP, but become coupled as the channel opens. The state dependence of energetic coupling that we observe and the conservation of NBD sequences among all ABC proteins, in particular of the residues involved in the hydrogen bond described here, provide strong experimental support to the idea that NBD dimerization drives CFTR channel opening using the same molecular mechanism used by its transporter relatives.

6. ATPase DRIVEN GATING IN CFTR'S TMDS

Although we do not have atomic resolution structures for CFTR's TMDS, low resolution structures of CFTR and of P-glycoprotein, a eukaryotic ABC exporter, revealed a striking structural similarity (Rosenberg *et al.* 2004), and a CFTR homology model built on the crystal structure of the bacterial exporter Sav1866 could be validated biochemically (Serohijos *et al.* 2008). So it is likely that CFTR's TMDS are structurally similar to those of more conformist ABC transporters. But what subtle structural changes could turn a transporter into a channel?

At no time point along the reaction coordinate of an active transporter can there be a pore open to both sides of the membrane, since this would allow dissipation of hard-won electrochemical gradients (Läuger 1980). Thus, a transporter needs to have the allocrite binding site, within the lipid bilayer, flanked on both sides by a gate: one gate limiting access to/from the cytoplasm and one gate limiting access to/from the external solution. As an example, an exporter binds the allocrite from the cytosolic side of the membrane (outer gate closed, figure 5a(i)), then, following a conformational change, releases it to the extracellular side (inner gate closed, figure 5a(iv)), and then in the unloaded state undergoes a further conformational change returning to its initial outward-facing conformation (figure 5a(iv)-(i)).

For a protein to function as an ion channel it must provide an aqueous pore through which the permeant ions flow. Because in this case ions move down their electrochemical gradient, and not against it, access to the permeation pathway need not be controlled by two gates. Since in crystals of the Sav1866 bacterial exporter, which have the NBDs in a tight dimer conformation, the TMDS have an 'outward-facing' conformation (Dawson & Locher 2006, 2007), it is simplest to imagine that CFTR's present working gate might be equivalent to a transporter's external gate (figure 5b). The formation of a tight NBD dimer would flip the TMDS into an outward facing conformation in which the structures homologous to a transporter's internal gate no longer prevent anion flow between the diffusion pathway and the cytosol (figure 5b(iii) and (iv)). Hydrolysis of the ATP bound at site 2, by triggering NBD dimer dissociation, would flip the TMDS to an inward facing conformation in which a functional external gate acts as an effective barrier between the external solution and the permeation pathway (figure 5b(i)).

Further observations suggest that conformational changes in CFTR's TMDS retain evidence of transporter ancestors. For the example exporter in figure 5a, the cycle of conformational changes must occur more frequently in the clockwise direction (central arrow). If one could look at these TMD conformational changes in isolation, an apparent violation of microscopic reversibility would be detected, suggesting coupling to a free energy releasing process. For an ABC exporter, this free energy is provided by the ATP hydrolysis cycle at the NBDs. It turns out that such a violation of microscopic reversibility can be detected by following single-channel CFTR current traces. Using heavily filtered traces three distinct conductances were observed, one closed (baseline current, C) and two open (a lower O1 and a higher O2) conductance levels (Gunderson & Kopito 1995). Following the inter-conversions between these levels, a time asymmetry was detected: the cycle C-O1-O2-C (57%) occurred much more frequently than the reverse C-O2-O1-C (4%). The remaining openings apparently followed linear pathways (C-O1-C, 27%, and C-O2-C, 12%), but likely many of these included non-resolved missed events. In various non-hydrolytic conditions, the O1-O2 transition virtually disappeared. Using a broader bandwidth, it was later shown that the two open conductance levels were caused by higher (O1) or lower (O2) frequencies of a fast flickery closure (Hennager *et al.* 2001). Analysis of 'burst type' distribution in saturating [ATP] revealed an apparent violation of microscopic reversibility, closely reproducing the previous findings. In the absence of ATP, with openings occurring approximately 150-fold less frequently than in saturating ATP, no time asymmetry was detected. Thus, in the presence of ATP, the conformational states C, O1 and O2 are not at thermodynamic equilibrium, but are kept at a steady state in which clockwise transitions (figure 5b) are more frequent than anticlockwise ones. The simplest interpretation of these observations is that the free energy needed to maintain the system away from equilibrium is, as in our

example ABC exporter, derived from the ATP hydrolysis cycle occurring at the NBDs.

7. CONCLUSION

The current evidence suggests that ATP hydrolysis drives CFTR channel gating. Why would a channel use a free energy source to drive conformational changes of its gates? Is this simply a vestigial, redundant aspect of CFTR channel function? Or could modulation of CFTR hydrolysis provide another sensitive level of regulation for activity, as has been shown for GTPases (Sprang *et al.* 2007)? Clearly we still have a long way to go before we really understand.

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