The role of nucleotide binding domain interactions in the gating of the CFTR ion channel

Ph.D. thesis

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Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR), the protein mutated in cystic fibrosis (CF) patients, is a member of the asymmetric C subfamily of adenosine-triphosphate(ATP)-binding cassette (ABC) proteins. Most ABC proteins are active transporters that move a variety of substrates across biological membranes. The substrate translocation pathway, formed by two transmembrane domains (TMDs), cycles between inward- and outward-facing conformations driven by an ATP hydrolysis cycle catalyzed by the two cytosolic nucleotide-binding domains (NBDs). ABC NBDs contain three highly conserved sequence motifs: the Walker A (GXXXXGKS/T) and B (ΦΦΦΦDE, with Φ representing hydrophobic residues), and the ABC signature motif (LSGGQR/K). In each catalytic cycle, ATP binding to the Walker motifs of both NBDs promotes formation of a tight head-to-tail NBD dimer that occludes two ATP molecules in composite interfacial catalytic sites formed by the Walker motifs of one NBD and the signature motif of the other. ATP hydrolysis is required to disrupt this dimer to allow nucleotide exchange and initiation of a new cycle. Members of the C subfamily share this overall architecture but show a marked asymmetry in the primary sequences of their two NBDs. Because of noncanonical substitutions in the Walker B and in other conserved motifs of the N-

terminal NBD (NBD1) and in the signature sequence of the C-terminal NBD (NBD2), the catalytic site containing these motifs (site 1) is inactive, leaving ABC-C proteins with a single catalytically active site (site 2), formed by Walker motifs of NBD2 and signature sequence of NBD1).

CFTR is the only member of the ABC-C family whose TMDs are known to form the pore of a Cl^- ion channel. In addition to the canonical ABC domains, CFTR contains a unique regulatory (R) domain, phosphorylation of which by cAMP-dependent protein kinase (PKA) is a prerequisite for channel gating. In phosphorylated CFTR, the pore opens to a burst upon ATP-induced dimerization of its NBDs and closes from a burst upon dimer dissociation after ATP hydrolysis at site 2. As evidence of such strict coupling between NBD and TMD movements, a hydrogen bond between the side chains of NBD2 Walker-A threonine (T1246) and an arginine (R555) adjacent to the NBD1 signature sequence was shown to form in open, but not in closed, channels. These two positions have co-evolved as a pair, and in crystal structures of nucleotide-bound ABC NBD dimers the analogous side chains form a hydrogen bond, the arginine serving as hydrogen donor and the threonine/serine side chain as acceptor. Furthermore, by disturbing proper spacing between the donor and acceptor sites, both shortening of the R555 and lengthening of the T1246 side chains (by single mutations R555K and T1246N,

respectively) prevent, whereas the double mutation R555K-T1246N restores, the formation of this stabilizing hydrogen bond.

In wild-type (WT) CFTR, each gating cycle (or burst of openings) is coupled to hydrolysis of one ATP molecule. Thus, the canonical catalytically active nucleotide-binding site cycles between dimerized prehydrolytic (state O_1), dimerized post-hydrolytic (state O_2), and dissociated (state C_1 and C_2) forms in a preferential $C_1 \rightarrow O_1 \rightarrow O_2 \rightarrow C_2$ sequence (cyclic gating scheme). ATP remains bound at CFTR's NBD1 for periods much longer (several minutes) than a channel gating cycle (~1 s), without being hydrolyzed. The high-affinity ATP analogue N^6-(2-phenylethyl)-ATP (P-ATP) speeds opening and slows closing of CFTR channels. The effect on opening rate appears instantaneously, suggesting that it is caused by P-ATP binding to the rapid-turnover site 2, whereas the onset of slowed closure follows with a delay of 30–50 s, consistent with ATP/P-ATP exchange occurring more slowly at degenerate site 1. Furthermore, because the nucleotide exchange rate at site 1 was affected by mutations in the NBD2 signature sequence, which completes site 1 only in a formed dimer, it was suggested that the NBD dimer only partially separates in closed channels, with the interface remaining closed around site 1 for several gating cycles. This notion obtained further support from thermodynamic studies that examined state-dependent changes in energetic coupling between pairs of residues on

opposing faces of the site-1 interface: the observed interaction pattern was consistent with the model of an “immobile” site 1, remaining closed throughout the gating cycle. Nevertheless, site 1 clearly does play a role in gating energetics, as perturbations at site 1 (K464A mutaton in NBD1, H1348A mutation in NBD2, or P-ATP instead of ATP) can profoundly alter channel gating kinetics.

WT CFTR channels open occasionally even in the absence of ATP, but it is unclear whether this reflects the occasional spontaneous formation of the NBD dimer, or occasional pore openings independent of NBD dimerization. The significance of this question goes beyond the scarcity of WT spontaneous openings. Indeed, the strictness of coupling between NBD and TMD movements is a strongly debated fundamental question, key for understanding the forces that drive the normal, ATP-dependent functional cycle of ABC proteins. Moreover, ivacaftor, the only potentiator drug to be approved so far for clinical use in CF patients, acts by promoting such spontaneous, ATP-independent, openings of G551D CFTR, the third most common disease mutant. However, mechanistic understanding of spontaneous gating has been limited by its vanishingly small open probability (P_o), far too low to be reliably quantified. Recently, mutations in the third intracellular loop (position 978, between TM helices 8 and 9), and at the cytosolic end of pore-lining TM helix 6 (position 355), were both found to strongly enhance spontaneous activity. Furthermore, these

’gain-of-function’ effects proved additive in the double-mutant P355A–K978C, shifting spontaneous P_o into a range where quantitative biophysical studies become tractable.

Objectives

Studying the mechanism of spontaneous gating

WT CFTR channels, in addition to their ATP-driven gating cycles, open occasionally even in the absence of ATP, but the molecular mechanism of such openings is unknown. The primary aim of our experiments was to determine whether these spontaneous openings, similarly to the ATP-driven gating cycles, reflect the formation of the NBD dimer, or whether they occur independently of NBD dimerization.

Studying the mechanism of conformational changes of the degenerate ATP binding site linked to channel gating

Despite the earlier suggestion that the interface remaining closed around site 1 for several gating cycles, this site clearly does play a role in gating energetics, as perturbations at site 1 can profoundly alter channel gating kinetics. The mechanism of burst prolongation of NBD2 signature sequence mutant H1348A and the structural perturbation of the ligand bound at site 1 (P-ATP instead of ATP) is not fully understood. To gain further insight into the energetic consequences of perturbations at site 1, and to dissect which gating steps are affected by the conformational changes at site 1, we examined in detail the effects of these perturbations on CFTR gating.

Methods

Molecular biology

Human WT CFTR in the pGEMHE plasmid served as template for mutants, which were created using the QuikChange kit (Agilent Technologies). The entire coding sequence of each construct was verified by automated sequencing (LGC Genomics). T7 polymerase was used for in vitro transcription (Mmessage kit; Ambion), and purified cRNA was stored at -80°C.

Isolation and injection of Xenopus laevis oocytes

Xenopus oocytes were extracted and treated with collagenase and stored at 18°C. To obtain expression levels appropriate for single-channel or macroscopic recordings, oocytes were microinjected with 0.1–10 ng of CFTR cRNA, and further incubated at 18°C for 1–3 days.

Excised inside-out patch-clamp recordings

For inside-out patch recordings, the pipette solution contained (mM) 136 NMDG-Cl, 2 $MgCl_2$, and 5 HEPES, pH 7.4 with NMDG. The bath solution was continuously flowing and contained (mM) 134 NMDG-Cl, 2 $MgCl_2$, 5 HEPES, and 0.5 EGTA, pH 7.1 with NMDG. For measurements on constructs bearing the K978C mutation the bath solution was freshly supplemented with 3 mM dithiothreitol to prevent

thiol oxidation of the cysteine side chain engineered into position 978. MgATP (Sigma-Aldrich) and P-ATP Na^+ salt (BIOLOG Life Science Institute) were used at final concentrations of 2 mM (or 10 mM for channels bearing the K1250A mutation) and 10 μM (or 50 μM for K1250A mutants). CFTR channels were fully activated by a 1-2-min cytosolic exposure to 300 nM of catalytic subunit of PKA (Sigma-Aldrich). All experiments shown were done in the partially dephosphorylated state after PKA removal, which remains stable over the time course of several minutes. Switching between various bath solutions was achieved using computer controlled electronic valves (HEKA); solution exchange time constant was 20-50 ms. Experiments were performed at 25°C. Unitary CFTR currents were recorded (Axopatch 200B; Molecular Devices) at a pipette holding potential of +80 mV (V_m = -80 mV), filtered at 2 kHz, and digitized at 10 kHz (Digidata 1322A, Pclamp10; Molecular Devices). As CFTR gating is largely voltage independent, macroscopic currents were recorded at membrane potentials between -20 and -80 mV.

Steady-state kinetic analysis of multichannel patches

Mean burst and interburst durations were extracted from steady segments of record with one to seven simultaneously open channels. Events lists were fitted with a simple model in which ATP-dependent slow gating is pooled into a closed-open scheme and ATP-independent

brief closures are modeled as pore-blockage events. Rate constants (r_{CO}, r_{OC}, r_{OB} and r_{BO}) of the resulting closed–open–blocked (C↔O↔B) scheme were extracted by a simultaneous maximum likelihood fit to the dwell-time histograms of all conductance levels while accounting for the filter dead time. Mean burst duration was then calculated as $(1/r_{OC})(1 + r_{OB}/r_{BO})$ and mean interburst duration was calculated as $1/r_{CO}$.

Burst analysis of single-channel patches

Open bursts from records with a single active channel were isolated by ignoring closures shorter than a cutoff. The distributions of the durations of bursts obtained in this way were fitted using maximum likelihood to either a single-exponential distribution or to the cyclic $C_1 \rightarrow O_1 \rightarrow O_2 \rightarrow C_2$ gating scheme with the slow rate $O_1 \rightarrow C_1$ fixed to 0. The improvement of the fit caused by introduction of the second free parameter was evaluated using the log-likelihood ratio test.

Analysis of macroscopic current relaxations

Macroscopic current decay time courses were fitted with single exponential functions using nonlinear least squares (pClamp10), and the closing rate was defined as the inverse of the fitted time constant.

Mutant cycle analysis

Mutant cycle analysis was used to obtain a measure of energetic coupling ($\Delta\Delta G_{int}$) between pairs of target structural elements. Perturbation-induced changes (between corners *i* and *j* of a mutant cycle) in free energy differences were calculated from the changes in P_o and rates (r) as $\Delta\Delta G = -RT\ln(P_{oj}/P_{oi})$ or $\Delta\Delta G = -RT\ln(r_j/r_i)$, and $\Delta\Delta G_{int}$ was defined as the difference between $\Delta\Delta G$ values along parallel sides of the mutant cycle. All $\Delta\Delta G$s are given as mean ± SEM. Because the numbers of observations (n) for each corner of the cycles were similar, SEMs were calculated using the mean value for n.

Statistics

Data are given as mean ± SEM of at least five measurements. Statistical significance was evaluated using Student's t test.

Results

Studying the mechanism of spontaneous gating

Whereas WT CFTR channel currents decline rapidly to baseline upon ATP removal, in patches containing P355A-K978C CFTR channels a readily measurable fraction of the chloride current resisted even prolonged removal of bath ATP ($P_{o(WT,ATPfree)}$ = 0.000053 ± 0.000021 (n = 5) vs. $P_{o(P355A-K978C,ATPfree)}$ = 0.15 ± 0.03 (n = 21)). This robust spontaneous activity of P355A-K978C channels in the absence of ATP allowed reliable sampling of the characteristics of spontaneous gating in microscopic patches. This double-mutant CFTR background allowed us to study, using the thermodynamic double mutant cycle formalism, changes in energetic coupling associated with spontaneous gating between the same two NBD-interface side chains previously used to demonstrate NBD dimer formation and disruption in CFTR channels opening and closing in ATP.

Mutating either the arginine at position 555 to a lysine, or the threonine at position 1246 to an asparagine, resulted in a large reduction in spontaneous P_o (0.034 ± 0.007 (n = 18) and 0.044 ± 0.009 (n = 19)), due to the loss in both single mutants of microscopic interactions, between the targeted side chain and the rest of the protein, that in WT channels combine to increase the stability of the open state.

If the side chains of residues R555 and T1246 did not interact in WT CFTR, or if their interaction were to remain unaltered during spontaneous gating, then any impact on the spontaneous channel activity of mutating either single position should be independent of the nature of the side chain found at the other position: that is, mutational effects at the two positions should add energetically. Any non-additive behaviour would signal gating-associated dynamic changes in the interaction energy between the R555 and T1246 side chains. In stark contrast to the single mutants, the double mutant R555K-T1246N displayed a high spontaneous P_o (0.11 ± 0.02 ($n = 20$)), comparable to that of the background construct. Thus, the effects on the spontaneous P_o of either single mutation depended strongly on the nature of the residue at the other position. The closed-open equilibrium constants (K) in the absence of ATP can be calculated from the spontaneous P_o values. A mutant cycle built on these K values revealed a significant ($p = 0.0004$) change of -2.90 ± 0.49 kT in R555-T1246 interaction energy between ATP-free closed- and open-channel states. This energetic coupling is consistent with the formation of a hydrogen bond, in the spontaneous open state in WT channels that is lost in both single mutants, but is restored in the double mutant. The inevitable implication of these findings is that the NBD dimer interface must tighten within site 2 upon pore opening, whether or not bound ATP is present.

Conformational transitions between stable open-channel (burst) and closed-channel (interburst) states of CFTR reflect passages across the transition ($T^{\ddagger}$) state, a high free-energy transient conformation. The rates of spontaneous opening and closure of each molecular channel type, obtained as the inverse of the mean interburst and burst durations, respectively, report on transition-state stability: mutation-induced fractional changes in spontaneous opening and closing rates quantitate changes in transition-state free energy relative to the closed and open ground states, respectively. Kinetic analysis revealed that the reduced spontaneous open probabilities of both single mutants R555K and T1246N were caused predominantly by a markedly reduced opening rate, which was restored in the double mutant. Consequently, a significant ($p = 0.0001$) negative value of -2.30 ± 0.46 kT was obtained for the change in coupling free energy between the target side chains upon reaching the transition state from the closed state. In contrast, closing rates were little affected by the mutations, yielding a value of $+0.62 \pm 0.34$ kT for $\Delta\Delta G_{int}$, not significantly different from zero ($p = 0.09$). These results imply that in WT channels the hydrogen bond between the R555 and T1246 side chains is already formed in the transition state for spontaneous opening, its strength being little further altered when the protein reaches the stable open state. Thus, the NBD dimer interface tightens

within site 2 already in the transition state for unliganded opening, just as it does during the opening of ATP-bound channels.

Studying the mechanism of conformational changes of the degenerate ATP binding site linked to channel gating

Kinetic analysis of patches containing a few channels revealed significantly higher P_o in P-ATP as compared with that seen in ATP, which was largely caused by approximately twofold longer mean burst durations together with a small reduction in interburst durations. The H1348A mutation also prolongs burst durations of CFTR channels gating in 2 mM ATP by approximately threefold, compared to WT.

To quantitatively compare the effects of our site-1 perturbations on the mechanism of closure, we studied their effects on the rates of non-hydrolitic and hydrolitic closure. The non-hydrolitic closing rates were modeled by macroscopic closing rates of CFTR mutants incapable of ATP hydrolysis (NBD2 Walker A mutant K1250A and NBD2 Walker B mutant E1371S). Both P-ATP and the H1348A mutation slowed current relaxations by two- to threefold compared to those observed for the two background constructs upon removal of ATP.

The duration of each open burst includes some time spent in a prehydrolytic open state (O_1) in which a nucleoside triphosphate is occluded at site 2, followed by a shorter time interval in a less stable

post-hydrolytic open state (O_2). Because the rate (k_{-1}) of nonhydrolytic closure (step $O_1 \rightarrow C_1$) is very slow, the overall mean burst duration is mostly determined by the rates of the $O_1 \rightarrow O_2$ and $O_2 \rightarrow C_2$ transitions (k_1 and k_2). The latter two rates can be estimated by fitting the cyclic gating scheme to the distributions of open burst durations using maximum likelihood. To determine which of these two rates is affected by P-ATP and the H1348A mutation, respectively, we recorded currents from patches containing a single active channel. Open burst events were isolated by burst analysis, pooled from several patches, and fitted by maximum likelihood. The histograms of burst durations were distinctly peaked for both WT channels gating in 10 μM P-ATP and H1348A channels gating in 2 mM ATP, consistent with a non-equilibrium gating cycle that involves nucleotide hydrolysis. The most striking difference between these distributions and that found earlier for WT channels gating in ATP was in the tail, which was distinctly longer in the presence of the site-1 perturbations, whereas the positions of the peaks were little moved. Accordingly, the maximum likelihood fit yielded two- to threefold slower k_1 values in the presence of P-ATP or the H1348A mutation compared with the earlier estimate for WT channels gating in ATP. In contrast, neither P-ATP nor the H1348A mutation seemed to dramatically affect the faster rate k_2.

Conclusions

Studying the mechanism of spontaneous gating

We showed that spontaneous pore openings are also linked to dimerization of the NBD interface around composite site 2, just as in the presence of bound ATP. The NBD dimer interface tightens within site 2 already in the spontaneous transition state.

The P355 and K978 amino acid side chains, affected by the gain-of-function mutations, experience some structural rearrangement upon reaching the transition state from either direction. This suggests that these sidechains are still in motion in the transition state. Thus, just as for ATP-bound channels, the pore is likely still closed in the high-energy evanescent transition state for spontaneous openings.

Comparison of the thermodynamics of gating for spontaneous activity of WT CFTR, and for ATP-driven activity of hydrolysis-incompetent mutant CFTR channels, shows that the binding of ATP stabilizes both the transition- and the open states, but the former to a lesser extent. This suggests a molecular rearrangement at the NBD interface when the channel relaxes from the transition state towards the stable open state. One possible explanation is that, in the transition state, site 1 has not yet reached its full open-channel-like conformation.

Studying the mechanism of conformational changes of the degenerate ATP binding site linked to channel gating

Both the H1348A mutation and ATP/P-ATP exchange decrease rates k_1 (step $O_1 \rightarrow O_2$) and k_{-1} (step $O_1 \rightarrow C_1$), i.e., increase the height of the thermodynamic barriers for the $O_1 \rightarrow O_2$ and the $O_1 \rightarrow C_1$ transitions. This suggests that the histidine sidechain and the phenylethyl group in site 1 experience some conformational changes in their environment during pore opening, and also during the hydrolysis of nucleotide bound at site 2. Thus, both of the examined perturbations selectively stabilize the prehydrolytic open (O_1) state.

Although the H1348A and the K464A mutations, that target the opposite sides of the degenerate nucleotide binding site, distort the free energy profile in different ways, both perturbations affect the rates of the $C_1 \rightarrow O_1$ and $O_1 \rightarrow C_1$ steps. This further supports the conclusion that a conformational rearrangement takes place at site 1 during channel opening. This rearrangement is still incomplete in the transition state: site 1 reaches its final open-like conformation only when the pore opens.

Bibliography of the candidate's publications

The PhD thesis is based on the following publications

Csanády L, **Mihályi C**, Szollosi A, Töröcsik B, Vergani P. (2013) Conformational changes in the catalytically inactive nucleotide-binding site of CFTR. J Gen Physiol. 142: 61-73.
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Other publications

Iordanov I, **Mihályi C**, Tóth B, Csanády L. (2016) The proposed channel-enzyme transient receptor potential melastatin 2 does not possess ADP ribose hydrolase activity. Elife 5. pii:e17600.
IF(2015): 8.282