Timing of motions in the CFTR channel protein during pore opening

Ph.D. Doctoral Dissertation

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Introduction

CFTR belongs to the ATP binding cassette (ABC) protein superfamily, whose members are involved in a broad variety of physiological, pharmacological and pathological processes. Although ABC proteins are active transporters whereas CFTR, alone, is a chloride ion channel, recent results suggest that a conserved molecular mechanism couples a cycle of conformational changes between nucleotide binding domains (NBDs) and transmembrane domains (TMDs) of all ABC proteins.

CFTR is a monomeric ABC protein built from two homologous halves, each comprising a TMD followed by a cytosolic NBD. However, CFTR is unique in that parts of its TMDs form a chloride-selective transmembrane ion channel. Both TMDs are built from six transmembrane α-helices. In CFTR, the two canonical ABC halves (TMD1-NBD1 and TMD2-NBD2) are linked into a single polypeptide by the unique cytosolic regulatory (R) domain, resulting in the following sequential domain order: TMD1-NBD1-R-TMD2-NBD2. The R domain is unrelated in its sequence to any other known protein, and is a target for phosphorylation by cAMP-dependent protein kinase (protein kinase A, PKA) at multiple serine residues. R-domain phosphorylation is a prerequisite for ATP-driven CFTR chloride channel activity.

In the presence of ATP NBD domains of all ABC proteins form a head-to-tail dimer with two ATP-binding sites formed at the shared interface by the Walker-A and B motifs (the "head") of one NBD, and the ABC signature motif (the "tail") of the opposing NBD. Hydrolysis of the bound ATP molecules is facilitates disruption of these stable dimers. CFTR is an asymmetrical ABC protein: only the composite binding site formed by Walker A and B motifs of NBD2 and the signature sequence of NBD1 (hereafter referred to as "site 2") is catalytically active; the other interfacial binding site (hereafter called "site 1") contains several non-canonical substitutions in its key motifs, and keeps ATP bound and unhydrolyzed throughout several NBD dimerization cycles. In CFTR NBD dimer formation initiates a burst of pore openings interrupted by brief closures, while dimer dissociation terminates the burst and returns the TMDs into a longlasting nonconducting (interburst) state. Thus, by analogy, it was suggested that in the bursting (‘‘open’’) state CFTR’s TMDs resemble the outward-facing, whereas in the interburst (‘‘closed’’) state they resemble the inward-facing conformation of ABC exporter TMDs Indeed, functional studies have largely confirmed that assignment. For wild-type (WT) CFTR gating is a unidirectional cycle: most openings are terminated by ATP hydrolysis rather than by the far slower pathway that involves separation of the ATP-bound stable NBD dimer without hydrolysis.

Transition states ($T^{‡}$), which determine the rates of functionally relevant conformational movements, are the highest-energy, shortest-lived conformations of proteins. For instance, for ion channels closed → open, and open → closed conformational transitions are so fast that they appear as single steps even in the highest time-resolution recordings. This implies that the time the channel protein spends in the $T^{‡}$ itself is on the sub-microsecond scale – in contrast to the long (milliseconds-seconds) intervals spent in comparatively stable open and closed ground states observable in single-channel recordings. Intractable by standard structural biological approaches, $T^{‡}$ structures can be studied using Rate-Equilibrium Free Energy Relationship (REFER) analysis, which reports on the relative timing of movements in selected protein regions during a conformational transition, such as a channel opening step. Structural perturbations (typically point mutations) in a given channel region often change channel open probability (P_o) by affecting the open-closed free-energy difference, but the extent to which the free energy of the barrier that must be traversed in opening or closing the channel is affected, depends on how early or late that region moves.

Rate-Equilibrium Free Energy Relationship (REFER) analysis is a thermodynamic approach which provides information on transition state structures of equilibrium reactions, but is not

applicable to non-equilibrium reactions. A REFER plot is a log-log plot of k_{co} (the rate of the closed → open transition) versus K_{eq} ($P_o/(1-P_o)$), for a series of structural perturbations introduced into a single region of a protein. Because the ratio Φ between the effects on transition-state and open-state stabilities is similar for perturbations of a single protein position, the slope of such a plot reports the value of Φ.

A region that moves early during opening will have already approached its open-state conformation in the overall transition state: a perturbation here thus affects the stability of the transition state to an extent similar to that of the open state (Φ=1). Thus, the height of the barrier for the opening transition changes, but the height of the barrier for the closing transition does not, so a perturbation in such a region impacts only opening, but not closing, rate. In contrast, a region that moves late during opening is still near its closed-state conformation in the transition state, thus, perturbations here that affect open-state stability do not affect transition-state stability. As a consequence, the height of the barrier for the closing transition changes, but the height of the barrier for the opening transition does not, so perturbations in such a region impact only closing, but not opening, rate. Finally, a region which is just on the move in the transition state, i.e., is in an intermediate conformation between its closed-like and open-like conformations, will be characterized by an

intermediate Φ value: by affecting the height of the barrier both for the opening and the closing transition, perturbations in such a region impact both opening rate, and closing rate, but in opposite directions.

REFER analysis has been extremely fruitful in mapping gating dynamics of the nicotinic acetylcholine receptor channel but is applicable only to equilibrium mechanisms, unlike that of WT CFTR. This drawback has so far hampered insight into the CFTR opening transition state. Although two REFER studies on CFTR have been published in the past, it has been demonstrated that this methodology can provide no useful information on the non-equilibrium cyclic mechanism which governs WT CFTR bursting.

To appropriately adapt the REFER technique to CFTR gating, a suitable construct is required, the gating of which approximates a simple equilibrium closed-open scheme. Hydrolysis-disrupting mutations truncate the normal CFTR gating cycle by blocking the ATP hydrolysis step, thereby reducing it to an equilibrium reaction. Among many known hydrolysis-deficient CFTR mutants D1370N appeared as an attractive choice for a REFER study, because its unusually fast non-hydrolytic closing rate (~0.5 s^{-1}) allows single-channel gating analysis, while ATP-dependence of channel opening was shown to be little changed. When studied in the presence of saturating ATP, the gating mechanism of such channels approximates a simple two-state

scheme: reversible transitions between states C_1 and O_1. A second problem which must be addressed for proper interpretation of REFER data is potentially altered phosphorylation-dependent regulation of mutant channels. Indeed, recent evidence suggests that the R domain regulates CFTR gating by directly interacting with the TMDs.

To selectively study the energetics of ATP-dependent gating, confounding effects of potentially altered TMD-R domain interactions must therefore be eliminated by using a CFTR construct lacking the R domain (ΔR). The modular ABC architecture offers a natural strategy to achieve that goal: functional CFTR channels can be obtained by coexpressing NBD1-TMD1 (residues 1-633) and NBD2-TMD2 (residues 837-1480) as separate polypeptides – a strategy nature employs for most prokaryotic ABC proteins. This "cut-ΔR" CFTR was shown to be active without prior phosphorylation but indistinguishable from (prephosphorylated) WT CFTR with respect to ATP-dependent gating. This minimalist channel – pared down to the essential ABC domains and containing the D1370N mutation in the NBD2 head – therefore appeared as an attractive candidate background for a REFER study aimed at obtaining meaningful biophysical information on CFTR channel opening dynamics.

Objectives

- To choose and characterize an appropriate background construct suitable for REFER studies
- To study the relative timing of the global conformational wave of the pore opening transition along the longitudinal protein axis

Methods

Molecular Biology

All constructs were made starting from pGEMHE-CFTR(837-1480) (C-half) and pGEMHE-CFTR(1-633) (N-half). First, mutation D1370N was introduced into C-half to generate C-half(D1370N). Mutations of target positions 275 and 348 were introduced into N-half, and those of target position 1246 into C-half(D1370N).

Heterologous expression: Isolation and injection of *Xenopus laevis* oocytes

Oocytes were isolated from anaesthetized *Xenopus laevis* and injected with cRNA. Single-channel and macroscopic recordings were made 12 hours–6 days after injection of 0.01–10 ng of cRNA.

Excised Patch Recording

Patch pipettes were pulled from borosilicate glass using a vertical pipette puller. Tips were heat-polished to a diameter of 1-2 μm (4-7 MΩ), or 5-6 μm (~1 MΩ), for single-channel or macropatch recordings, respectively. Patches were excised and transferred to a flow chamber, where the cytoplasmic surface was continuously superfused with standard bath solution containing various test

substances. In the case of chloride-based bath solutions the bath electrode (Ag/AgCl pellet) was immersed directly into the flow chamber. In experiments that involved replacement of the bath anion the electrode was placed in 100 mM KCl and connected to the flow chamber by an agar bridge (4% agar in 100 mM KCl). CFTR channels were activated by 10 mM Magnesium-ATP (pH = 7.1 with NMDG), added into a specified bath solution. The holding potential was at -80 mV (-100 mV for formate currents), and currents were recorded at a bandwidth of 2 kHz and digitized at 10 kHz. Single-channel patches were identified as very long (typically 15 min – 1 hr) recordings without superimposed channel openings.

REFER analysis

For each target site, five substitutions were chosen so as to perturb structure as little as possible, but still sufficiently alter P_o for reliable Φ estimation. In general, side chains of various sizes, polarities and charges were chosen. Experiments were done at saturating [ATP] determined individually for each construct, to ensure that the channel opening step is not rate limited by ATP binding.

Mean interburst (τ_{ib}) and burst (τ_b) durations obtained from steady-state burst-analysis of microscopic currents in 10 mM ATP were used to define opening (k_{CO}) and closing (k_{OC}) rates as $1/\tau_{ib}$ and

$1/\tau_b$, respectively; K_{eq} was then defined as k_{CO}/k_{OC}. Brønsted plots (ln k_{CO} vs. ln K_{eq}) were fitted by linear regression.

Results

Gating of cut-ΔR(D1370N) did not require prior exposure to PKA but remained strictly ATP-dependent with an apparent affinity for ATP of 288 ± 27 μM. Just as for WT, cut-ΔR, and D1370N CFTR channels, mean τ_b of cut-ΔR(D1370N) proved largely ATP-independent: the time constant of macroscopic current relaxation following ATP removal, a measure of τ_b in zero ATP, was 1,342 ± 72 ms; which is similar to the steady-state τ_b of 1,526 ± 301 ms for single channels opening and closing in saturating (10 mM) ATP.

The perturbations introduced into site-2 NBD interface position 1246, by replacing the native threonine with valine, proline, cysteine, asparagine, and alanine, dramatically reduced P_o by prolonging τ_{ib}, i.e., by slowing channel opening rate ($k_{CO}=1/\tau_{ib}$). In comparison, τ_b, and hence closing rates ($1/\tau_b$), were less affected. Correspondingly, the REFER plot for position 1246 yielded a steep slope of $\Phi=0.97\pm0.19$, indicating that this position moves very early during the pore opening conformational transition.

Perturbations introduced into coupling helix 2 of the NBD2-TMD transmission interface at position 275 caused modest changes in P_o but in both directions. Here, we substituted tyrosine for phenylalanine, glutamate, lysine, leucine, and serine. Kinetic analysis revealed a clear tendency for opposing effects on channel

closing and opening rates, both contributing about equally to changes in P_o: lengthened τ_b was mostly associated with shortened τ_{ib} and (in Y275L) shortened τ_b with lengthened τ_{ib}. Changes in opening rate ($1/\tau_{ib}$) again reflected changes in rate k_{CO}. These coupled changes resulted in a REFER plot with an intermediate slope of $\Phi = 0.50 \pm 0.13$, indicating that position 275 has not yet reached its final open-like position in the opening transition state.

To perturb intra-pore position 348, we systematically replaced the native methionine with isoleucine, lysine, cysteine, asparagine, or alanine. Except for the lysine substitution, perturbations at position 348 all dramatically reduced P_o, and this effect was in every case due to speeding of closing rate (reduction in τ_b), with little change in opening rate ($1/\tau_{ib}$). This led to a REFER plot with a small slope of $\Phi=0.20\pm0.12$, indicating that this pore region still resembles its closed-state conformation in the opening transition state.

Intriguingly, replacement of chloride with nitrate was shown to affect CFTR gating, suggesting that interactions of permeating anions with residues lining the "filter" region of the open pore energetically contribute to open-state stability. Thus, replacement of chloride with other permeant anions might be viewed as a structural perturbation of the "selectivity filter". In addition to documented reductions in unitary conductance, perturbations of the filter by

replacement of chloride with the following selected permeant anions all affected gating: nitrate and bromide which bind more tightly in the pore increased P_o, while formate which binds less tightly decreased it. Importantly, both gating effects primarily reflected changes in τ_b, i.e., in rate k_{OC}, with smaller changes in τ_{ib}. The slope of the REFER plot constructed from these data yielded $\Phi = 0.28 \pm 0.03$, similar to that of position 348.

It is clear that the strikingly different Φ values for our three target positions defined a clear spatial gradient along the protein's longitudinal axis from cytoplasm to cell exterior: the very high Φ value of ~1.0 for site-2 NBD interface position 1246 stands in stark contrast to the low Φ value of ~0.2 for both the intra-pore position 348 and for another Brønsted plot of the pore region, obtained from anion substitution studies. Compared to these extreme Φ values, Φ~0.5 for the NBD-TMD interface position 275 appears intermediate. Hence, we found clear directionality of motion along the longitudinal protein axis, and identified an opening transition-state structure with the NBD dimer formed but the pore still closed. Thus, strain at the NBD/pore-domain interface, the ΔF508 mutation locus, underlies the energetic barrier for opening.

Conclusions

- CFTR cut-ΔR(D1370N), obtained by coexpression of CFTR segments 1-633 and 837-1480(D1370N) in *Xenopus laevis* oocytes, is active in the absence of phosphorylation and gates in a strictly ATP-dependent manner.
- In the presence of saturating ATP the ATP-dependent slow gating process of CFTR cut-ΔR(D1370N) is reduced to a two-state equilibrium mechanism, suitable for REFER analysis.
- Mutations of position 1246 in the NBD2 Walker A motif (composite site 2) affect opening, but not closing rate of cut-ΔR(D1370N).
- Mutations of position 275 in coupling helix 2 affect both opening and closing rate of cut-ΔR(D1370N), but in opposite directions.
- Mutations of position 348 in TM6 affect primarily closing, but not opening rate of cut-ΔR(D1370N).
- Substitution of permeating chloride ions for nitrate, bromide, or formate affect primarily closing, but not opening rate of cut-ΔR(D1370N).
- The pore opening conformational change is initiated at the site-2 NBD interface, and is propagated along the longitudinal protein axis towards the pore.

- In the transition state for pore opening the site-2 NBD interface is already tightened, but the pore is still closed, creating strain at the NBD-TMD interface which contains disease locus F508.

Publication

The PhD thesis is based on the following publication:

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