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CONTROL OF THE CFTR CHANNEL'S GATES

Paola Vergani, Claudia Basso, Martin Mense, Angus C. Nairn^{*,†}, and David C. Gadsby
Laboratory of Cardiac/Membrane Physiology, The Rockefeller University, New York, NY 10021, U.S.A

^{*} Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021, U.S.A

[†] Dept of Psychiatry, Yale University, New Haven, CT 06519, U.S.A

SYNOPSIS

Unique among ABC (ATP-binding cassette) protein family members, CFTR (cystic fibrosis transmembrane conductance regulator), encoded by the gene mutated in cystic fibrosis patients, functions as an ion channel. Opening and closing of its anion-selective pore are linked to ATP binding and hydrolysis at CFTR's two nucleotide binding domains, NBD1 and NBD2. Isolated NBDs of prokaryotic ABC proteins form homodimers upon binding ATP, but separate after hydrolysis of the ATP. By combining mutagenesis with single-channel recording, nucleotide photolabeling, and sulfhydryl-specific crosslinking methods on intact CFTR molecules, we relate opening and closing of the channel gates to ATP-mediated events in the NBDs. In particular, we demonstrate that two CFTR residues, predicted to lie on opposite sides of its anticipated NBD1-NBD2 heterodimer interface, are energetically coupled when the channels open but are independent of each other in closed channels. This directly links ATP-driven tight dimerization of CFTR's cytoplasmic nucleotide binding domains to opening of the ion channel in the transmembrane domains. Evolutionary conservation of the energetically coupled residues in a manner that preserves their ability to form a hydrogen bond argues that this molecular mechanism, involving dynamic restructuring of the NBD dimer interface, is shared by all members of the ABC protein superfamily.

Keywords

CFTR; ATP binding and hydrolysis; single-channel kinetics; ABC protein

INTRODUCTION

Cystic fibrosis is the most common lethal genetic disease in Caucasian populations. It is caused by mutations in the *CFTR* gene, which encodes an integral membrane protein that belongs to the ATP binding cassette (ABC) superfamily [1]. CFTR is unique among ABC proteins in that it functions as an ion channel, its two transmembrane domains forming an anion-selective permeation pathway. Opening and closing (gating) of the channel is subject to complex regulation. First, opening requires phosphorylation, by cAMP-dependent protein kinase, of multiple serines in CFTR's regulatory or "R" domain [2]. Second, once the channels are phosphorylated, gating is controlled by binding and hydrolysis of ATP. The molecular mechanisms underlying the ATP-dependent gating of pre-phosphorylated channels will be our focus here.

ATP binding occurs at CFTR's two nucleotide-binding domains (N-terminal NBD1 and C-terminal NBD2). Crystal structures of isolated CFTR NBD1 [3–5] revealed a fold that was similar to that (reviewed in [6]) of other ABC protein NBD subunits. As in other monomeric crystals, contacts between nucleotide and protein occur at residues belonging to the conserved Walker motifs in the ATP binding core [7] - or “head” - subdomain, while the ABC-specific “LSGGQ” signature sequence, within the α -helical “tail” subdomain, is relatively distant from the bound nucleotide. However, growing biochemical [8–13] and structural [14–16] evidence suggests that prokaryotic NBDs can form “head-to-tail” dimers. In this arrangement, the signature sequence in the tail of one subunit interacts with the γ -phosphate of the ATP molecule bound to the head of the partner subunit, so that two composite ATP binding sites are formed. In tightly juxtaposed dimeric crystals [14,16] the two ATP molecules sandwiched at the interface mediate a large part of the intersubunit interactions. Thus dimers, formed following ATP binding, would come apart only after hydrolysis and product release, leading to the hypothesis that in ABC transporters formation/dissociation of tight NBD dimers might couple the hydrolytic cycle to functionally important conformational changes in the associated transmembrane domains [8,10,14]. For CFTR, biochemical studies [17,18,19] and single-channel kinetic analysis [20] have led to the proposal that opening of the Cl^- channel is coupled to formation of an NBD1/NBD2 intramolecular dimer, and dissociation of this dimer - hastened by hydrolysis at NBD2 - closes the channel (Fig. 1). Here we present some experimental evidence supporting this interpretation.

RESULTS AND DISCUSSION

Sulfhydryl-specific crosslinking establishes a direct physical interaction between the two NBDs

We have shown that coexpression, in *Xenopus* oocytes, of two separate half CFTR molecules (amino acids 1–633 and 634–1480) results in functional channels with essentially wild type (WT) channel properties [21]. Similarly, coexpression of a WT N-terminal 1–633 fragment and a cysteine-free C-terminal 634–1480 fragment results in functional CFTR channels, which can be expressed at levels sufficiently high for biochemical analyses. To test the hypothesis that the two NBDs of CFTR form an NBD1/NBD2 dimer with two composite catalytic sites at the dimer interface, we introduced single cysteines either into NBD1, in the N-terminal half molecule, or into NBD2 in the cysteine-free C-terminal half molecule, or into both NBDs. Sulfhydryl-specific bifunctional crosslinkers BMH (bis-maleimidoethane) and BMOE (bis-maleimidoethane) formed crosslinks between cysteines introduced at positions 549 (in the NBD1 tail, within the ABC signature sequence) and 1248 (in the NBD2 head, within the Walker motifs) and the crosslinked product could be detected by Western blot analysis. These observations demonstrate that, in functioning CFTR, a head-to-tail NBD1/NBD2 intramolecular heterodimer is present for at least some fraction of the protein's duty cycle.

ATP binding precedes channel opening

To determine at which stage of the gating cycle ATP binding is required, we measured the [ATP] dependence of single-channel kinetic parameters. Following pre-phosphorylation, patches were exposed to a range of [ATP], each test interposed between bracketing periods at the reference [ATP] of 5 mM. Reducing [ATP] did not affect average open burst duration. However, at low [ATP], the closed dwell time - the average time spent in the closed state between bursts, inversely related to opening rate - was increased. For WT, a simple hyperbolic equation with an apparent dissociation constant of $\sim 50 \mu\text{M}$ ATP describes the dose-response curve of opening rate as a function of [ATP]. 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 which of the two composite sites in the NBD1/NBD2 dimer is involved in channel opening, we introduced mutations at residues seen to interact directly with the bound nucleotide in the solved crystal structures, in the head of either NBD1 (K464A) or NBD2 (D1370N). For either mutant, opening rate was reduced at low [ATP], but this impairment could be largely overcome by increasing [ATP]. Thus binding at either site can be made rate limiting for channel opening. The simplest interpretation for these results is that binding to both head subdomains must occur before the transition to the open state becomes energetically favourable [20].

Hydrolysis at NBD2 allows fast channel closure

Closing rate is not strongly affected by [ATP]. On the other hand, CFTR, like other ABC proteins, is an active ATPase [22], and interfering with the hydrolytic cycle, through addition of either inorganic vanadate [23,24] or non-hydrolysable nucleotide analogs ([21,24–26]), slows channel closing [17,20]. While the effect of such compounds can be mimicked by introducing mutations at key catalytic positions in the composite NBD2 site [20,26–30], equivalent mutations at the corresponding residues in the NBD1 site do not significantly affect closing rate [20,27–29,31]. These data suggest that the open channel, with two bound ATP molecules, is thermodynamically stable, but that hydrolysis of the nucleotide bound at NBD2 allows rapid exit from the open state, effectively rendering the opening transition kinetically reversible.

Occlusion of ATP at the NBD1 site

The interaction between CFTR and nucleotides can be studied using the photoactive nucleotide 8-azido-ATP, which, upon irradiation with UV light, can form a covalent link to residues within the binding pocket [32], allowing quantification of nucleotide associated with the protein at the moment of UV irradiation. When CFTR-expressing membranes were incubated with radio labelled 8-azido-ATP and washed extensively in nucleotide-free solution before UV irradiation, it was found that bound nucleotide dissociated from the CFTR polypeptide only after prolonged post-incubation washes at 30°C (time constant of radioactivity loss ~ 15 min). Experiments in which the two halves of CFTR were coexpressed as separate molecules identified this extremely slow turnover site - at which the intact ATP remains “occluded” for minutes - as the NBD1 site [17]. The observation that non-conservative substitutions are present in the WT CFTR sequence at key catalytic positions in the NBD1 head and in the NBD2 tail [17] strengthens the latter conclusion and suggests that CFTR’s composite NBD1 site has lost its catalytic role.

Channel opening and tight NBD dimerization

To test the idea that opening of CFTR channels could be coupled to formation of a tight NBD1/NBD2 dimer we investigated the interaction between residues predicted to lie on opposite sides of the dimer interface. We first studied the functional consequence of sulfhydryl-specific crosslinking in CFTR channels containing introduced cysteines in the NBD1 tail (S549C) and in the NBD2 head (S1248C), in a background similar to that used for the biochemical studies described above. Patch-clamp studies monitoring channel-closing rate upon removal of ATP suggest that oxidative crosslinking arrests channels in an open-burst state, from which they can fully close only following reduction by DTT [18]. Thus a tight NBD1/NBD2 dimer in the cytosol would correspond to a predominantly open-channel state.

We next analysed the interaction between R555, three residues after the “LSGGQ” signature sequence in the NBD1 tail, and T1246, within the Walker motif in the NBD2 head. We chose to study this pair of residues based on the statistical coupling analysis [33] we performed on a sequence alignment comprising more than 10,000 NBD sequences (<http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00005>). The analysis (Fig 2) indicated that this pair of positions had co-evolved. Changes in side chain at one position had occurred in

concert with changes at the other, so that most sequences contain two side chains capable of forming a hydrogen bond which can correctly position the two α -carbons at a ~ 10 Å distance: either an Arg-Ser (or an equivalent Arg-Thr), or a Lys-Asn pair (Fig. 2B).

To study energetic coupling between this pair of residues we applied mutant cycle analysis [34,35]. The WT, the two single mutants (in our case R555K and T1246N) and the double mutant (R555K/T1246N) form the corners of a thermodynamic cycle. If the two residues are independent, then the effects of mutations in WT and in mutant background will be the same. Any non-zero difference between mutation-linked changes on parallel sides of the cycle signifies - and quantifies - energetic coupling between the two target residues (see Fig. 3, below).

We first analysed the energetics of ATP binding, which occurs on closed channels, using the mutant cycle formalism to examine coupling in the closed state. Because the step linking the ATP-bound closed state to the open state (C_2 to Open in Fig. 1) is relatively slow, the ATP-binding reaction reaches a steady state that is probably not far from equilibrium. The apparent dissociation constant obtained from [ATP] dependence of opening rate is therefore a reasonable estimate for the real dissociation constant for the ATP binding reaction at NBD2. 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 Arg or Lys. The effects of the Thr to Asn mutation in WT and mutant (R555K) background are thus similar, yielding a negligible energetic coupling between the two target residues ($\Delta\Delta G_{\text{int(unbound-bound)}} = 0.3 \pm 0.5$ kT). This indicates that any coupling is similar in the closed states before (C_1 state in Fig. 1) and after (C_2 state) ATP binding to NBD2. Because in all ATP bound structures, both monomeric [36–38] and dimeric [8,14,16], 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 an interdomain hydrogen bond involving T1246. We therefore conclude that there is no significant energetic coupling between T1246 and R555 in either closed state, neither before nor after ATP binding.

We next analysed 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 (Fig. 3). The significant energetic coupling ($\Delta\Delta G_{\text{int(opening)}}^\ddagger = -2.7 \pm 0.5$ kT) we obtain is 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.

We then 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, at saturating [ATP], the gating scheme in Fig. 1 is reduced to a simple closed-to-open equilibrium. 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, [39]) strongly destabilized the open state with respect to the closed one. The same mutation, however, when introduced in the non-hydrolytic background which also contained the R555K mutation, did not significantly alter the closed-to-open equilibrium. The calculated coupling energy ($\Delta\Delta G_{\text{int(open-closed)}} = -2.4 \pm 1.0$ kT) is again 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 [30].

The state dependence of energetic coupling we observe - the target residues are not coupled in closed states, but become coupled as the channel opens - provides strong experimental support to a mechanism involving dynamic NBD dimerization [8,10,14]. The conservation of NBD

sequences, in particular of the structural underpinnings of the hydrogen bond described here, suggests that most ABC transporters may share this molecular mechanism.

CONCLUSIONS

Interpreting our results in the light of recent advances in understanding of ABC protein structure and function [6], we propose a model that relates ATP binding and hydrolysis at CFTR's cytosolic NBDs to gating of the ion-channel pore (Fig. 1). The model is sufficient to account for most observations, but it is possible that states not included in the scheme (e.g. open mono-liganded channels, [40,41]), may be visited, albeit rarely. ATP binding at the NBD1 and NBD2 heads would lead to conformational changes favouring formation of an intramolecular NBD1/NBD2 head-to-tail dimer. Such structural rearrangements at the cytosolic NBDs would be transmitted to the transmembrane domains, ultimately resulting in opening of the Cl⁻ permeation pathway. Hydrolysis of the ATP bound at the active NBD2 composite site, with ensuing loss of the γ -phosphate's stabilizing contributions, would allow NBD dimer dissociation and reversal of the channel-opening conformational changes in the transmembrane domains.

Abbreviations

ABC	ATP binding cassette
CFTR	cystic fibrosis transmembrane conductance regulator
NBD	nucleotide binding domain

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Figure 1.

Cartoon illustrating proposed mechanism of ATP-dependent regulation of the CFTR channel gate. Formation of an intramolecular heterodimer between NBD1 (in green) and NBD2 (in blue) is coupled to conformational changes in the transmembrane domains (in dark grey) that open the permeation pathway.

Figure 2.

Statistical coupling analysis detects co-evolution between two positions corresponding to CFTR's R555 (putative hydrogen bond donor) and T1246 (putative hydrogen bond acceptor).

A. Top, Schematic representation of the total multiple sequence alignment and subsets of alignments obtained by “fixing” the side-chain at the donor site (one grey line represents approximately 400 NBD sequences; coloured rectangles indicate the side-chain present either at donor, blue shades, or at acceptor, red shades, position). Below: Side-chain distribution at acceptor position in total multiple sequence alignment and in each different subset. **B.** Schematic representation of pairs of side-chains present at highest frequency (Modified from [25]).

Figure 3.

Mutant cycle analysis using activation free energies for the opening reaction. Coupling between R555 and T1246 increases as the channel approaches the open state. **A.** Representative records from WT, single mutants R555K and T1246N, and double mutant R555K T1246N, activated with 300 nM PKA and 5 mM ATP. Changes in the activation energy barrier ($\Delta\Delta G^{\ddagger}$) caused by each side-chain substitution are given beside each arrow ($\pm$ st.dev.). **B.** Bar chart shows summary of mean closed dwell times ($\pm$ SEM) (Modified from [25]).