

Anion Channels

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Three Dimensional Reconstruction of CFTR Chloride Channel Using Single Particle Analysis

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The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is a membrane integral protein that belongs to an ATP-binding cassette superfamily. Mutations in the CFTR gene cause cystic fibrosis (CF) in which salt, water and protein transports are defective in various tissues. Here we have expressed wild-type human CFTR as a FLAG-fused protein in HEK293 cells heterologously, and purified it in three steps: anti-FLAG and wheat germ agglutinin affinity chromatographies and size exclusion chromatography. The stoichiometry of the protein was analyzed using various biochemical approaches, including chemical cross-linking, blue-native PAGE, size exclusion chromatography, and EM observation of antibody decorated CFTR. All these data support a dimeric assembly of CFTR. Using 5,039 automatically selected particles from negatively stained EM images, the 3D structure of CFTR was reconstructed at 2 nm resolution assuming a two-fold symmetry. CFTR presumably in a closed state is shown to be an ellipsoidal particle with dimensions of $120 \times 106 \times 162 \text{ \AA}$. It comprises a small dome-shaped extracellular and membrane-spanning domain, and a large cytoplasmic domain with orifices beneath the putative transmembrane domain. EM observation of CFTR/anti-R-domain antibody complex confirmed that two R-domains located around the bottom end of the larger oval cytoplasmic domain. This is the first clear 3D-structural presentation of CFTR molecule in 'tail to tail' dimeric configuration.

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A Triad of Residues F1296-N1303-R1358 in NBD2 of CFTR is Involved in ATP-driven Gating

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Despite the general agreement that dimerization of CFTR's two Nucleotide Binding Domains (NBDs) drives channel opening, little is known about how nucleotide binding to individual NBDs promotes dimer formation. We investigated the allosteric interaction between three NBD2 residues, F1296, N1303 and R1358, because statistical coupling analysis reveals coevolution of these positions. Considering frequently occurring pairs in the multiple sequence alignment, we chose mutations F1296S and N1303Q for building a mutant cycle. The mutations had no significant effect on the apparent ATP affinity measured in inside-out macropatches. However, we observed elevated ATP-independent activity ($P_{o,bas}$) in F1296S/N1303Q. $P_{o,bas}$ was 0.0048 ± 0.0024 , 0.0034 ± 0.0007 , 0.013 ± 0.0027 , and 0.115 ± 0.028 for WT, F1296S, N1303Q, and F1296S/N1303Q, respectively. A thermodynamic mutant cycle built on these values implies a change in coupling ($\Delta\Delta G = -5.74 \pm 1.45 \text{ kJ/mol}$) between positions 1296 and 1303 upon channel opening in the absence of ATP. To study the coupling between these two positions in the presence of ATP, but under equilibrium conditions, we introduced the same mutations into a non-hydrolytic background (K1250R). The high ATP-independent basal activity caused by the F1296S/N1303Q double-mutation was preserved in non-hydrolytic background. In addition, the mean burst duration of F1296S/N1303Q/K1250R in saturating ATP - estimated from the monoexponential macroscopic current decay time course following ATP removal - was more than 3-fold longer ($31.34 \pm 4.99 \text{ s}$) than that of K1250R ($8.25 \pm 0.58 \text{ s}$), F1296S/K1250R ($7.77 \pm 0.48 \text{ s}$), or N1303Q/K1250R ($9.09 \pm 0.95 \text{ s}$), predicting a change in coupling ($\Delta\Delta G = -2.95 \pm 0.48 \text{ kJ/mol}$) between positions 1296 and 1303 as the channel closes from the ATP-bound open conformation. Thus, the interaction between F1296 and N1303 is important to stabilize the open state but is not an important determinant of ATP affinity. The N1303-R1358 interaction is under current investigation.

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Using Correlation Analysis To Predict Pairs Of Energetically Coupled Residues At The NBD-TMD Interface In CFTR

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CFTR, whose failure causes cystic fibrosis, is a chloride channel, which belongs to the ATP binding cassette (ABC) transporter family. Like other

ABC proteins, CFTR consists of two halves, each containing a cytosolic nucleotide-binding domain (NBD1, NBD2) and a transmembrane spanning domain (TMD1, TMD2). ATP binding and hydrolysis at the NBDs control the CFTR gate, presumed to be in the TMDs. It remains unclear precisely how the NBD/TMD coupling is mediated.

We used correlation analysis to identify possible pairs of energetically coupled residues which might mediate direct interactions between the NBDs and the TMDs. First, since CFTR belongs to a subgroup of ABCs, which contain two very divergent NBDs, we constructed an alignment containing only similar, asymmetric transporters. We implemented 5 different correlation algorithms. The major difficulty with correlation analysis is the prevalence of false positives due to non-independence of sequences resulting from evolutionary constraints. Only one algorithm corrects for non-independence by referring to an inferred phylogenetic tree, resulting in a smaller output. The other 4 methods assume independence and assign a score to every possible pair of alignment positions. Only pairs scoring in the top 1% were included.

To select candidate pairs at the NBD/TMD interface we looked for pairs found with more than one algorithm and separated by less than $\sim 15 \text{ \AA}$ on a Sav1866 based homology model of CFTR. We also checked the distributions of amino acids at the two positions on the inferred phylogenetic tree, to eliminate correlations clearly due to evolutionary branching. We identified several pairs which are close to sites which can be cross-linked after cysteine substitution. Functional characterization of the effects of mutations on single-channel kinetics is underway.

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Two Distinct Gating Cycles of CFTR Chloride Channels

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CFTR channels, at maximally effective [ATP], switch between open and closed states $1-2 \text{ s}^{-1}$. Pyrophosphate (PPI), applied with ATP, slows down the gating cycle by locking the channel in a stable open state ($\tau_o \sim 30 \text{ s}$). PPI alone, applied immediately following closing of ATP-opened channels, locks open the channel with the same open time constant. However, the open state induced by PPI long after ATP removal ($> 2 \text{ min}$) assumes a lifetime of 1.5 s, indicating the presence of two different closed states with distinct responses to PPI. By altering the duration of ATP removal and measuring the response of closed channels to PPI, we estimated the lifetime of the closed state (C^*) that enters the lock-open state to be $\sim 30 \text{ s}$. Since the lifetime of the C^* state can be modulated by N^6 -phenylethyl-ATP (P-ATP), a high-affinity ATP analog, or by mutations that lower the ATP binding affinity at NBD1, we propose that one ATP molecule remains tightly bound at NBD1 during this closed state. As the trapped ATP molecule should survive for numerous gating cycles before being replaced by a second ligand, we carried out single channel experiments where the perfusion solution containing 2 mM ATP ($r_{open} = 3.33 \pm 0.19 \text{ s}^{-1}$; $\tau_{open} = 235 \pm 19.8 \text{ ms}$) was directly (dead time $\sim 40 \text{ ms}$) switched to one with 50 μM P-ATP. The P_o of the channel increases in two steps. The channel opening rate was immediately increased ($r_{open} = 4.88 \pm 0.45 \text{ s}^{-1}$) upon solution exchange, while the open time was not prolonged until $\sim 40 \text{ s}$ after the application of P-ATP ($\tau_{open} = 386.7 \pm 25.2 \text{ ms}$). This result indicates two gating cycles; one is solely driven by fast ATP binding/hydrolysis in NBD2 while another involves slow dissociation of ATP in NBD1.

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CFTR: Differential Reactivity, State-dependent Accessibility And Blocker Occlusion Of Cysteines Substituted For Adjacent Residues In TM6

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We and others have identified residues in transmembrane segment six (TM6) of the CFTR chloride channel where substituted cysteines react with externally applied, polar, thiol-directed probes. A scan of TM6 showed that the profile of reactivity differed for channel-permeant reagents, such as $[\text{Au}(\text{CN})_2]^-$, and channel-impermeant reagents, such as MTSET^+ and MTSES^- . A cysteine at 338 (T338C) reacted with both channel-impermeant and -permeant probes while a cysteine at 337 (F337C) was unreactive toward channel-impermeant probes but reacted with $[\text{Au}(\text{CN})_2]^-$. The reaction rate of $[\text{Au}(\text{CN})_2]^-$ was 200 times faster with T338C than with F337C. Furthermore, the reactivity of F337C was highly dependent on the activation state of the channel, being greatly reduced prior to channel activation. T338C exhibited much less pronounced dependence on the activation state. We also compared the ability of a presumed open-channel blocker, GlyH-101, to occlude reactions at the two cysteines. F337C was effectively protected from reaction toward $[\text{Au}(\text{CN})_2]^-$,