many positively charged residues which could curtail the sliding due to attractive interactions with the negatively charged DNA. By single-molecule spectroscopy we have observed a fluorescently-labeled sliding clamp (pol III β subunit or β clamp), loaded onto freely diffusing, single-stranded M13 circular DNA annealed with fluorescently labeled DNA oligomers of up to 90 bases. We use solution-based single molecule fluorescence resonance energy transfer (FRET) experiments. We analyze the results using burst analysis and purified fluorescence correlation spectroscopy (PFCS). We find that the diffusion constant for the β clamp diffusing along DNA is on the order of 10^{-14} m^2/s_at least three orders of magnitude less than that for diffusion through water alone. We also find that the β clamp remains at the 3′ end in the presence of *E. coli* single-stranded binding protein (SSB). These results may imply that the clamp not only acts as a tether, but also a placeholder.

Platform AZ: Anion Channels

2616-Plat The ClC-0 Chloride Channel Is a "Broken" Cl^-/H^+ Antiporter

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ClC-0 is the quintessential CLC chloride channel. Previously, ClC-0 gating was shown to be a non-equilibrium process [1]. It was proposed that the source of energy driving this process is the chloride electrochemical gradient. However, although this explanation was qualitatively satisfying, it failed to quantitatively account for the data. Since there appeared to be no other source of energy in the system, this created a mystery. Inspired by the recent discovery that some of the CLC proteins are Cl^-/H^+ antiporters [2, 3, 4] we revisited this problem by testing the hypothesis that proton movement powers the non-equilibrium gating in ClC-0. Using single-channel recordings, we show that the proton electrochemical gradient quantitatively accounts for the observed non-equilibrium gating. These results indicate that ClC-0 gating catalyzes proton transport and suggest that ClC-0 is a "broken" Cl^-/H^+ antiporter [5], in which one of the conformational states has become leaky for chloride ions. Currently, we are conducting experiments to examine specifically the conformational changes associated with the gating.

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2617-Plat R-helix Couples Movements in the C-terminal Domain to the Pore of CLC-0

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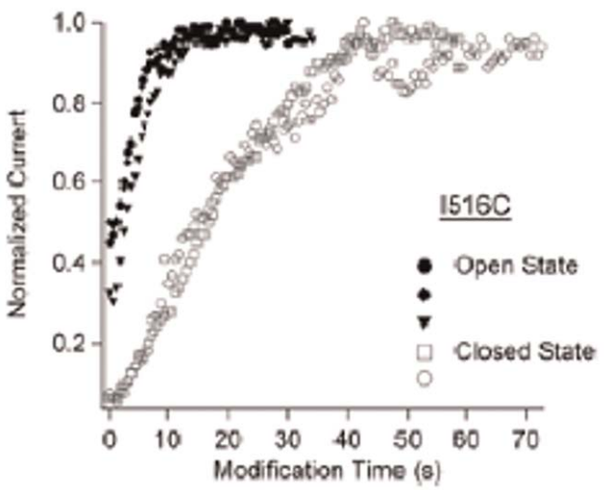
The common (slow) gate of CLC-0 generates long silent periods in single-channel recordings. Our previous FRET measurements suggest that common gating involves a large movement of the C-terminal domain. How do movements in the intracellular region affect chloride permeation through the membrane-spanning core? To try to answer this question, our current study focuses on R-helix, a pore-forming structure that directly links the C-terminal domain to the chloride permeation pathway. When the common gate is locked in its open state (with C212S), cysteine mutations in R-helix have little effects on fast gating. However, these same mutations strongly affect the kinetics as well as equilibrium of common gating in the wildtype channel background. Furthermore, the modification rate of R-helix cysteines is strongly dependent on the state of the common gate. These results indicate that R-helix has an important role in common gating.



2618-Plat Thermodynamics Of Anion Binding To a ClC Transporter

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CLC-ec1 is a Cl^-/H^+ antiporter of the CLC family. In a transporter substrate binding triggers a cascade of conformational changes whose result is ion translocation across the membrane. Two parameters characterize a transport system: its transport rate and the affinity of substrate. Little is known about the thermodynamic properties of substrate binding to CLC-ec1. The crystal structures show that Cl^- can bind to three sites that define a pathway through the protein. In the WT the inner and central sites are occupied by Cl^- while the external one is occupied by E148. We used Isothermal Titration Calorimetry to measure Cl^- binding to CLC-ec1 and found it has a K_d of ~0.5 mM, ~6 times lower than previously reported. Additionally, we found that Br^- and NO_3^- have K_d's ~2.2 mM and ~5 mM respectively. This selectivity preference closely mirrors the permeability sequence estimated by electrophysiological measurements. To determine the Cl^- affinities of the individual sites we used mutants that destabilize one or more binding sites like E148A, Y445A and E148A/Y445A. We found that Cl^- binds to the E148A

mutant -where the external site is also occupied by Cl^-- with a K_d of ~15 μM; ~50-fold more strongly than to the WT. In contrast, we do not detect Cl^- binding to the Y445A mutant, in which only the inner site is occupied by Cl^-, indicating a $K_d > 20$ mM. The double mutant E148A/Y445A displays a slightly reduced Cl^- affinity compared to the WT, with a K_d~1 mM. These experiments suggest that Cl^- binding to each site is strongly dependent on the occupancy of the other sites, indicating interactions between the bound Cl^- ions. Surprisingly, the tightest binding is observed with all 3 sites occupied by Cl^-, suggesting positive rather than negative cooperativity.

2619-Plat Zinc Ion Inhibits Human Clc-4 Transporter Currents In Xenopus Ooctyes

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The CLC family of chloride channels and transporters is a functionally diverse group of proteins important in a wide range of physiological processes. ClC-4 and ClC-5 are localized to endosomes and seem to play roles in the acidification of these compartments. These proteins were recently shown to function as Cl−/H+ antiporters, however relatively little is known about the detailed mechanism of CLC-mediated Cl−/H+ antiport, especially for the mammalian isoforms. We have attempted to identify molecular tools that might be useful in probing structure-function relationships in these proteins. Here, we record currents from human ClC-4 expressed in Xenopus oocytes and find that Zn2+ inhibits these currents with an apparent affinity of around 50 μM. Although Cd2+ has a similar effect, Co2+ and Mn2+ do not inhibit ClC-4 currents. Zn2+-mediated inhibition is minimally voltage dependent, suggesting an extracellular binding site for the ion; of nine candidate external residues tested, mutations of 3 consecutive histidine residues located in single extracellular loop significantly reduce the effect of Zn2+, with one of these making a larger contribution than the other two. Zn2+ inhibition is dramatically affected by manipulations that alter transport properties of ClC-4, including varying permeant ions as well as mutating the "gating glutamate", suggesting the involvement of a heretofore unexplored part of the protein in the transport process.

2620-Plat CFTR: How The "Transporter-turned-channel" Went From Transporter To Channel

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CFTR is a member of the ABC Transporter Superfamily, an ancient family of proteins found in all phyla. In nearly all cases, ABC proteins form transporters that couple the hydrolysis of ATP to the transmembrane movement of substrate. In contrast, CFTR is best known for its activity as an ATP-dependent chloride channel, where binding of ATP leads to channel opening and hydrolysis leads to channel closure. Near the predicted cytoplasmic end of transmembrane helix six (TM6) lies R352. Our structure/function experiments identified an interaction between R352 and D993 (in TM9), consistent with the formation of a salt bridge. Charge-destroying mutations at either site led to instability of the open state, while nearly wildtype-like behavior was observed in R352E/D993R-CFTR. These data suggest that R352 and D993 may interact during channel opening, thus stabilizing the open state. Such an interaction should disrupt the alternating-access mechanism of a transporter protein. We compared CFTR protein sequences to those of a variety of other ABC Transporters, which identified the MRP proteins as the closest mammalian paralogs, and used statistical analysis of the CFTR-MRP multiple sequence alignment to identify the specific domains and residues most likely to be involved in the evolutionary transition from transporter to channel activity. The amino terminal transmembrane domain (TM1-6) showed the strongest signal of CFTR-MRP functional divergence. R352 was identified as being involved in CFTR functional divergence, by virtue of being both CFTR-specific and conserved among all CFTR orthologs, while D993 was found to be absolutely conserved between CFTR and MRP. These data suggest that CFTR channel activity evolved, at least in part, due to the ability to lock the permeation pathway into a channel-like conformation, open at both ends, via formation of a salt bridge.

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2621-Plat Opening Of CFTR Mutants By Cadmium

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CFTR (Cystic Fibrosis Transmembrane conductance Regulator) is a chloride channel regulated by phosphorylation and gated by ATP hydrolysis. CFTR consists of two membrane-spanning domains, two nucleotide binding domains (NBD1 and NBD2) and a regulatory domain. There is growing evidence that NBDs form a head-to-tail dimeric structure with two ATP-binding sites buried in the dimer interface. Furthermore, it is widely accepted that NBD dimerization leads to channel opening. However, how the NBD dimer formation is coupled to channel opening is unclear. We found that although micromolar cadmium (Cd^{2+}) had little effect on wild type CFTR, it opens the G551D-CFTR channel, a disease-associated mutant with defective ATP-dependent gating. This effect of Cd^{2+} is not seen with calcium up to 1 mM. We hypothesize that Cd^{2+} can facilitate channel gating of G551D-CFTR through bridging D551 and a cysteine residue in CFTR. Supporting evidence includes:

(A) Pretreatment of the G551D-CFTR with cysteine modification reagent, MTSEA, protects the channel from Cd^{2+} activation;
(B) G551D mutant channels with all cysteine residues mutated (G551D/C-less) can no longer respond to Cd^{2+};
(C) Dose response studies show that 10 μM of Cd^{2+} give rise to about half of the maximum effect. This $K_{1/2}$ is decreased to 5 μM when G551 is mutated to cysteine, suggesting the formation of a high-affinity binding site for Cd^{2+} with multiple cysteines;

(D) An even lower $K_{1/2}$ of 1 μM is observed for the S549C mutation.

Surprisingly, the candidate cysteine residue is not located at NBD2, since Cd^{2+} still activates G551D/NBD2-C-less channel, in which all cysteine residues in NBD2 are converted to serines. These results suggest a gating mechanism independent of NBD dimerization. Identifying the involved Cd^{2+} interacting cysteine residues will likely provide insight into understanding the coupling mechanism of CFTR gating.

2622-Plat Studies at CFTR's composite site 1

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CFTR, whose failure causes cystic fibrosis, is a chloride channel, which belongs to the ATP binding cassette (ABC) transporter family. ABC proteins are characterised by their ability to bind and hydrolyse ATP. Like other ABC transporters, CFTR consists of two halves, each containing a cytosolic nucleotide-binding domain (NBD1, NBD2) and a transmembrane spanning domain (TMD1, TMD2). The NBDs are highly conserved, containing 3 motifs, the Walker A and Walker B motifs, and the signature sequence. Gating in CFTR is thought to be driven by the dimerisation of the two NBDs, in a head-to-tail configuration. Two functionally distinct composite ATP-binding sites are formed by the Walker A and B motifs of one NBD and the signature sequence of the other NBD. It is thought that binding and hydrolysis of ATP at the NBD2-composite site trigger channel opening and channel closure respectively, but the function of the NBD1 composite site is less clear.

We investigated the role of two residues, T460 and L1353 on either side of the NBD1- composite binding site, which have been shown to interact in other ABC composite sites. Surprisingly, neither of the mutations T460S or L1353M, appear to have any effect on normal gating. Measurements from patches containing up to 10 channels, show that the mean burst duration for T460S (347 ± 170ms) and L1353M (367 ±183ms) is not significantly different from WT (409 ±107ms) and noise analysis indicates no effect on open probability, P_o. Furthermore there is no change in apparent affinity for ATP. However, preliminary results indicate that the ability of pyrophosphate to lock open channels is reduced in the T460S mutant, consistent with this residue being important for stabilising the NBD1-NBD2 dimer, at least in non-hydrolytic conditions.

2623-Plat Kiss-and-run Gating Of CFTR Chloride Channels

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There is convincing evidence that opening of the CFTR chloride channel is associated with dimerization of CFTR's two NBDs (NBD1 and NBD2). We have shown previously that ATP binding to NBD2, but not NBD1, is critical for channel opening by ATP. Contrary to wild-type (WT) CFTR, which usually opens for hundreds of milliseconds, hydrolysis-deficient mutants can open for minutes. Interestingly, we observed that WT-CFTR occasionally shows long-lasting openings similar to that of hydrolysis-deficient mutants. This observation can be explained if the bound ATP at NBD2 dissociates before it is hydrolyzed, rendering the channel closure through a non-hydrolytic mechanism. This hypothesis predicts that mutants with a lower binding affinity of ATP to NBD2 will exhibit more long-lasting openings. For WT-CFTR, removal of ATP in excised inside-out patches results in a fast current decay that can be fitted with a single exponential function (τ = 533 +/− 104ms n = 9). Similar results were seen with W401G and Y1219W mutants (τ = 553 +/− 110 ms for W401G and τ = 726 +/− 115 ms for Y1219W). However, mutations including Y1219G, Y1219I and Y1219F with lower ATP binding affinity at NBD2 show different kinetic behavior. Current decay upon removal of ATP can be better fitted with a double exponential function. The time constants of the slow phase are 31.2 +/− 3.3 s, 28.7 +/− 1.63 s and 13.45 +/− 1.46 s for Y1219G, Y1219I and Y1219F respectively. In addition, the fraction of the slow component follows the same order as the degree of changes in ATP affinity characterized previously (i.e, Y1219G > Y1219I > Y1219F). These data are consistent with the idea that once ATP binding at NBD2 leads to channel opening, this ATP molecule does not have to stay there to maintain the open state.

Platform BA: Ryanodine Receptors

2624-Plat The Open and Closed Conformations of the RyR by CryoEM

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The intracellular calcium release channel/ryanodine receptor isoform of skeletal muscle (RyR1) controls a key step in the process that links nerve stimulation with muscle contraction. Using cryo electron microscopy of frozen-hydrated solubilized RyR1s followed by single-particle image processing we previously determined the basic architecture of RyR1's transmembrane domain in closed state. Our results (Samso, Wagenknecht et al., 2005) indicated that it has an ion gate defined by a four-helix bundle, a selectivity filter, and an intervening central cavity similar to the canonic architecture determined for the K^+ channel in the closed state.

To understand how the ion pathway is modified upon RyR1's opening, we now determine the 3D structure of RyR1 in open state. A highly uniform open state was obtained by using FKBP12 and BZ95 (Wong, Brackney et al., 1997), to induce long-lasting open states with a P_o of 0.99. The open state 3D reconstruction, obtained by single-particle cryoEM, displays a resolution that is sufficient to distinguish secondary structure within the transmembrane assembly. The comparison of RyR1 in the closed and open states reveals a conformational change that involves most of RyR1's domains. The changes in the cytoplasmic domains appear coordinated with changes in the central fourfold axis along the putative ion pathway. In particular, the diameter defined by the alpha helices in the region that we attribute to the ion gate increases in the open state. This conformational change is of the same order of that seen with opening in K^+ channels and appears to be pertinent to account for ion gating.

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