association of the distal C-terminus significantly enhanced VDI of $Ca_V1.2$ channels and that the effects of different β-subunits on VDI were small relative to the distal C-terminus. These findings expand the functional repertoire assigned to the distal C-terminal domain and support an important role in regulating Ca influx via its control of VDI.

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29-Plat C-terminal Splicing Fine-tunes Typical Low-voltage Activation Of Cav1.3 L-type Calcium Channels

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Low-voltage activation of Cav1.3 L-type Ca2+ channels (LTCCs) controls excitability in sensory and central neurons as well sinoatrial node pacemaking. Cav1.3 mediated pace-making determines neuronal vulnerability of dopaminergic striatal neurons affected in Parkinsons' disease.

We have previously identified an intrinsic distal C-terminal modulator (CTM) that regulates voltage- and calcium-dependent gating of Cav1.4 LTCC. Given the high homology in the C-terminus we hypothesize that a short Cav1.3 (Cav1.3S) splice variant that lacks potential corresponding CTM would also be affected in its gating properties. We therefore expressed either the full length (Cav1.3L) or the short Cav1.3S splice form together with β3 and α2δ-1 in tsA-201 cells and determined their biophysical properties using whole-cell patch-clamp technique. Ca2+ currents through Cav1.3S activated at more negative voltages (V0.5act: Cav1.3S: −12.9±0.8mV, n=15; Cav1.3L: −0.8±0.8mV, n=16: $p<0.0001$), inactivated faster (fraction of peak current remaining after 250ms: Cav1.3S: 0.13±0.02, n=6; Cav1.3L: 0.46±0.05, n=5; $p<0.01$) and showed more significant CDI ($p<0.01$). Window current was shifted to more negative potentials. The short Cav1.3 splice variant is significantly expressed in human brain, retina and heart and several mouse brain regions, cochlea and eye as identified in qualitative and quantitative RT-PCR experiments. Moreover, we have identified the Cav1.3 CTM within the last 116 amino acids.

These experiments revealed a novel mechanism of channel modulation in Cav1.3 LTCCs enabling alternative splicing to tightly control channel gating. The absence of the CTM in short splice forms leads to Cav1.3 channels activating at even lower voltages than previously reported for Cav1.3L very likely to fine-tune Cav1.3 function at negative voltages as required for the modulation of neuronal firing behaviour and sinoatrial node pacemaking.

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30-Plat Amino-terminal CaM binding Site (*NSCaTE*) Specifies Contrasts in Spatial Ca^{2+} Selectivity of Ca^{2+}-dependent Inactivation (CDI) in Ca_V1 versus Ca_V2 Channel Clades

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Ca^{2+}-dependent regulation of Ca^{2+} channels is orchestrated by a single calmodulin (CaM), constitutively associated with the carboxy termini of Ca_V1-2 channels. Remarkably, Ca^{2+} binding to each lobe of CaM can trigger distinct forms of regulation on a host channel. Furthermore, whereas the C-lobe responds to the intense local Ca^{2+} signal generated by the home channel ('local Ca^{2+} selectivity'), the N-lobe somehow favors the weaker cumulative Ca^{2+} signal generated by multiple Ca^{2+} sources over larger spatial domains ('global Ca^{2+} selectivity). The latter selectivity is vital for spatially coordinated Ca^{2+} feedback, yet the malleability of this detection mode has been uncertain. Here, we show that such N-lobe selectivity can be transformed by a Ca^{2+}/CaM binding site (*NSCaTE*: N-Terminal Spatial Ca^{2+} Transforming Element), situated within the amino termini of certain channels. For Ca_V2 channels, *NSCaTE* is absent and N-lobe CDI exhibits a global selectivity. By contrast, in $Ca_V1.2$ and $Ca_V1.3$, where *NSCaTE* is naturally present, N-lobe CDI adopts a local selectivity. The transforming potential of *NSCaTE* is linked to Ca^{2+}/CaM binding, as deletions impacting binding revert N-lobe CDI to a global profile. Additionally, three *NSCaTE* residues are crucial for Ca^{2+}/CaM interaction, and perturbations of these reveal a tight correlation between Ca^{2+}/CaM binding and spatial selectivity. Finally, *NSCaTE* effects are transferable, as tethering *NSCaTE* to $Ca_V2.1$ and $Ca_V2.2$ channels changes their N-lobe CDI towards local selectivity. This effect of an amino terminal element is unprecedented, given the preponderance of known structural determinants for CaM regulation on channel carboxy termini. Indeed, the amino-terminal position of *NSCaTE* argues that Ca^{2+}/CaM can bridge the carboxy and amino termini of $Ca_V1.2$/$Ca_V1.3$ channels, expanding on a theme wherein CaM can crosslink separate parts of a single molecule.

Platform C: Structure Function of Membrane Transport

31-Plat How Subunit Coupling Produces the Rotary Motion In F_1-ATPase: Insights from Simulation

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F_oF_1-ATP synthase manufactures the energy "currency", ATP, of living cells. The soluble F_1 portion, called F_1-ATPase, can act as a rotary motor, with ATP binding, hydrolysis, and product release, inducing a torque on the gamma subunit. A coarse grained plastic network model has been used to show at a residue level of detail how the conformational changes of the catalytic beta subunits act on the

gamma subunit through repulsive van der Waals interactions to generate a torque that drives unidirectional rotation, as observed experimentally. The simulations suggest that the calculated 85° substep rotation is driven primarily by ATP binding and that the subsequent 35° substep rotation is produced by product release from one beta subunit and a concomitant binding pocket expansion of another beta subunit. The results of the simulation agree with single molecule experiments and support a tri-site rotary mechanism for the operation of F_1-ATPase under physiological condition.

32-Plat ATP Hydrolysis Induced Domain Opening in the Nucleotide Binding Domains of ABC Transporters

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ATP-binding cassette (ABC) transporters are among the most ubiquitous transporters in all species ranging from bacteria to human. Functional ABC transporters require at least four domains: two transmembrane permease domains (TMDs) that provide a transmembrane pathway for substrate translocation, and two highly conserved nucleotide binding domains (NBDs) to provide energy from ATP hydrolysis. Using crystal structures of the NBDs of maltose permease (MalK) we have performed molecular dynamics simulations on different combinations of ATP- and ADP-bound states of the NBDs. Our simulations show that the first elements to respond to ATP hydrolysis are the Q-loops of the NBDs, which immediately separate after the conversion of ATP to ADP and P_i. This initial separation triggers a large-scale dissociation of the helical subdomain from the core domain of the other NBD, which results in the opening of the active site between the two NBDs. We also discovered that dimeric NBDs tend to adopt asymmetric nucleotide bound conformations even when they are both occupied by ATP; the most significant variation between the two NBDs is located at the Q-loop. The side chain of Gln82 in the ATP state serves as a metal binding ligand for ATP-bound Mg^{2+}, while in the ADP state the Q-loop becomes disordered and loses its close connection to the substrate. In addition, Tyr84 in one of the two Q-loops frequently makes contact to the other Q-loop across the dimer interface, which may be the origin of the discrete conformations between the two Q-loops and could prevent nucleotide hydrolysis in one of the active sites. Based on these results, we suggest that achieving at an optimal orientation of the Q-loop toward the ATP-Mg^{2+} site might be the rate limiting step in ATP hydrolysis.

33-Plat The Cyclopiazonic Acid Inhibitory Complex Reveals New Insights into the Calcium Pump Transport Cycle

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The sarcoplasmic reticulum Ca^{2+}-ATPase is essential for calcium reuptake in the muscle contraction-relaxation cycle. We have determined the structures of the E2-P state with bound cyclopiazonic acid (CPA) and magnesium fluoride at 2.65 Å resolution, and the E2 state with bound CPA and ADP at 3.4 Å resolution (Moncoq, K. *et al.* (2007) J. Biol. Chem. *30*:9748). The structures reveal that CPA inhibits Ca^{2+}-ATPase by blocking the cytosolic calcium access channel. In the E2(CPA) structure, ADP is bound to a novel site that is an adenine-recognition motif commonly found in ATP-dependent proteins. While two nucleotide binding sites have been postulated for the P-type ATPases, this is the first structural evidence for a second site. Docking of ATP into this site positions the γ-phosphate within 5 Å of Asp^{351}. This suggests a mechanism for activation where the γ-phosphate of ATP is positioned first, followed by a rotation of the adenine ring from an initial recognition site to the nucleotide binding site. The implications of nucleotide binding on the Ca^{2+}-ATPase transport cycle will be discussed.

34-Plat Conformational Fluctuations Of The Ca^{2+} ATPase In The Native Membrane Environment. Effects Of pH, Temperature, Substrates And Inhibitors

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Digestion with proteinase K or trypsin yields complementary information on conformational transitions of the Ca^{2+} ATPase (SERCA). Distinct digestion patterns are obtained with proteinase K, revealing interconversion of E1 and E2, or E1~P and E2-P states. The pH dependence of digestion patterns shows that, in the presence of Mg^{2+}, conversion of E2 to E1 occurs even in the absence of Ca^{2+}, as H^+ dissociate from acidic residues. Mutational analysis demonstrates that E309 and E771 (within the empty Ca^{2+} binding sites I and II) are required for stabilization of E2 in the absence of Ca^{2+}. In all cases, a further transition produced by Ca^{2+} binding to E1 (i.e., E1.2Ca^{2+}) is still needed for catalytic activation. Following ATP utilization, H^+/Ca^{2+} exchange is involved in the E1~P.2Ca^{2+} to the E2-P transition, whereby alkaline pH is a limiting factor. Complementary experiments on digestion with trypsin exhibit high temperature dependence indicating that, in the E1 and E2 ground states, the ATPase conformation undergoes strong fluctuations related to internal protein dynamics. The fluctuations are tightly constrained by ATP binding and phosphoenzyme formation, and this constraint must be then overcome by thermal activation and substrate free energy to allow enzyme turnover. In fact, the E1~P.2Ca^{2+} to E2-P + 2Ca^{2+} transition entails an input of approximately 8 – 9 Kcal per cycle (-2RTln ($10^3M^{-1}/10^6M^{-1}$)). Therefore, a substantial portion of the ATP free energy is utilized for conformational work related to the E1~P.2Ca^{2+} to E2-P transition. The functional relevance of conformational transitions is demonstrated by the effects of inhibitors.

35-Plat Ion Pathway Through The Na/K Pump

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Thermodynamically uphill exchange of three cytoplasmic Na ions for two extracellular K ions by the Na/K pump, with one ATP hydrolyzed, results from conformational changes that allow access to the pump's ion binding sites from one side of the membrane and then the other. Formally, the pump behaves like an ion channel with two tightly coupled gates, one each side of the binding sites, that are constrained to open and close alternately. The marine toxin, palytoxin, disrupts the coupling between the gates, allowing both to sometimes be open, so temporarily transforming the pump into an ion channel. Palytoxin thus permits application to the Na/K-ATPase of methods developed for examining the ion pathway through ion channels. Small charged, or neutral, methanethiosulfonate (MTS) reagents probe cysteine targets introduced one at a time throughout the pump's transmembrane (TM) segments. Changes in the flow of Na ions through palytoxin-bound pump-channels signal reaction. We previously found that residues in TM segments 4 and 6 contribute to an ion pathway characterized by a wide vestibule open to the extracellular surface that penetrates deep into the Na/K-ATPase where it narrows and leads to a cation-selectivity filter comprising conserved acidic residues involved in cation coordination. We have now extended the scan and find that reactive positions line a pathway from one side of the membrane to the other, bounded by TM1, TM2, TM4, and TM6, that passes through the Na/K-pump equivalent of cation binding site II in SERCA Ca-ATPase. We found no evidence for MTSET reaction with cysteines at any of 20 contiguous positions along TM5. These data provide a snapshot of the ion pathway through the Na/K pump which, on the basis of structural homology, is likely to be similar in related P-type ATPases, such as Ca and H/K pumps. [HL36783]

36-Plat Structural Mechanism Of Ion And Substrate Specificity In Sodium Coupled Secondary Transporters

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The x-ray structures of LeuT and Glt , a bacterial homologue of Na+/Cl–dependent amino-acid transporters, provides a great opportunity to better understand the molecular basis of monovalent cation selectivity and specific substrate uptake in ion-coupled transporters. Both proteins possesses two or more ion-binding sites, that are highly selective for Na+. Extensive all-atom free energy molecular dynamics simulations of the LeuT and Glt transporters embedded in an explicit membrane are performed at different temperatures and various occupancy states of the binding sites to dissect the molecular mechanism of ion selectivity. The absolute (standard) binding free energies of three different amino acids to LeuT and mutant forms of LeuT are calculated using a novel algorithm proposed by Wang et al. (Wang, Deng and Roux, BJ, 2006) Computations are based on the combination of extensive molecular dynamics simulations with free energy perturbations done in the explicit solvent. To enhance conformational sampling we employed a set of restraining potentials. We report the absolute hydration energies for several amino acids in zwitter-ionic form, provide detailed decomposition per-amino acid contributions to the ligand stabilization in the binding site and analyze effect of different point-mutations, hypothesized to be important for site specificity. In this work, we demonstrate that binding of different ligands alters the collective dynamics of neutral residues in the binding pocket (F253, Q250) and charged side-chains (R30-D404) forming the extracellular (EC) gate in LeuT. The important role of hydrogen bonding and hydrophobic interactions for EC gating dynamics in the LeuT transporter are discussed.

37-Plat Dynamic movement of helices I and V of LacY

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X-ray structures of the lactose permease of *Escherichia coli* (LacY) and the C154G mutant, which binds sugar with high affinity but catalyzes little or no transport, have been obtained. Both structures exhibit two pseudo-symmetrical 6-helix bundles surrounding a large hydrophilic cavity wide open on the cytoplasm side, but with no access to the binding site from the periplasmic side. Helix V contains 3 residues important for sugar binding. The periplasmic sides of helices I and V exhibit close contacts with helices VII and VIII, respectively, sealing the cavity from the outside. Clearly, the periplasmic side must open during turnover to allow access of sugar to the binding site. To begin to investigate dynamics in this region of LacY, 8 functional paired-Cys replacements were constructed with an engineered factor Xa protease cleavage site in loop IV/V. Although none of the mutants form spontaneous disulfides in right-side-out membrane vesicles, 3 pairs (28C/154C, 28C/158C and 29C/158C) form disulfides in the presence of Cu(II)-phenanthroline (Cu/Ph), and 5 pairs (24C/154C, 28C/154C, 28C/158C, 27C/158C and 29C/158C) crosslink with the homobifunctional crosslinker MTS-1-MTS. Moreover, in mutants 24C/154C, 25C/158C and 29C/158C, MTS-1-MTS-mediated crosslinking exhibits no effect on lactose transport, although complete crosslinking is obtained with mutant 29C/158C. Strikingly, however, with mutants 24C/154C, 28C/154C, 28C/158C and 29C/158C, disulfides catalyzed by Cu/Ph abrogates transport activity, indicating that effects observed at the periplasmic ends of helices I and V do not reflect ridge-body movements. Furthermore, formation of all disulfides and crosslinks is enhanced in the presence of sugar or by introduction of mutation C154G, suggesting that both ligand binding and the C154G mutation induce similar movement leading to an increase in the population of a conformer(s) with closer packing between helices I and V.

38-Plat Ammonium Transport Mechanisms in the Amt/Rh Protein Family

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Transport of ammonium across cellular membranes is a fundamental physiological process. Although ammonium is highly toxic to animals, it is the preferred source of nitrogen for most microorganisms. Ammonium transport is mediated by a family of ubiquitous membrane proteins (Amt), found in all domains of life and homologous to animals Rhesus (Rh) proteins. Based on the X-ray structure of the *E. coli* AmtB transporter, it was concluded that the conduction mechanism involves the single file diffusion of electro-neutral ammonia (NH_3) molecules, excluding the presence of water molecules in the narrowest portion of the pore (Khademi et al., Science 2004). This lead the Amt/Rh proteins to be generally considered as ammonia conducting channels. However, this model neglects the fact that X-ray diffraction experiments have shown electronic density in the pore lumen for crystals grown in both presence and absence of ammonium salt (Zheng et al., PNAS 2004). Furthermore, free energy calculations suggest that the hydrophobic pore of AmtB is able to stabilize a file of water molecules at positions in excellent agreement with the experimental electronic density (Lamoureux et al., Biophys. J. 2007). The possible presence of water molecules in the pore lumen of AmtB calls for the reassessment of the so far accepted permeation model. Functional experiments on plant ammonium transporters and rhesus proteins suggest a variety of permeation mechanisms including the passive diffusion of NH_3, the antiport of NH_4^+/H^+, the transport of NH_4^+, or the cotransport of NH_3/H^+ (Javelle et al., J. Struct. Biol. 2007). In the light of some recent functional and simulation studies on the AmtB transporter, we discuss these mechanisms and illustrate how they can be reconciled with the available high resolution X-ray data.

Platform D: Membrane Proteins I

39-Plat Determining the Conductance of the SecY Protein Translocation Channel for Small Molecules

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The channel formed by the SecY complex must maintain the membrane barrier for ions and other small molecules during the translocation of membrane or secretory proteins. We have tested the permeability of the channel using planar bilayers containing reconstituted purified E. coli SecY complex. Wild type SecY complex did not show any conductance for ions or water. Deletion of the "plug", a short helix normally located in the center of the SecY complex, or modification of a cysteine introduced into the plug, resulted in transient channel openings; a similar effect was seen with a mutation in the pore ring, a constriction in the center of the channel. Permanent channel opening occurred when the plug was moved out of the way by disulfide bridge formation. These data show that the resting channel on its own forms a barrier for small molecules, with both the pore ring and the plug required for the seal; channel opening requires movement of the plug (Saparov et al., 2007).

References

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40-Plat Role of Histidine Protonation in the pH-Triggered Membrane Insertion of Diphtheria Toxin T-Domain

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Diphtheria toxin T-domain inserts into the lipid bilayer and translocates the catalytic domain across the endosomal membrane in response to acidification. Similar to other self-inserting proteins (colicins, annexin B12), T-domain requires negatively charged lipids for proper insertion, suggesting a possible role for cationic residues. We hypothesize that protonation of histidines plays an important role in

(A) destabilizing the aqueous state of the T-domain and
(B) assisting in interfacial refolding via charge-charge interaction with anionic lipids.

We test this hypothesis by replacing one or several His's either with neutral Gln's or charged Arg's. We examine the folding and thermal stability of the mutants in solution using circular dichroism and test their membrane activity in an ANTS/DPX vesicle leakage assay. We also used several fluorescence quenching techniques to characterize the compactness of the fold in solution and topology of the membrane-inserted state. Our data indicate that the T-domain can tolerate multiple substitutions without losing its native-like CD-appearance and pore-forming activity. Substitutions of all 6 His's, or either of the N-terminal (223, 251, 257) or C-terminal (322, 323, 372) His's with Gln's slowed down leakage kinetics. Multiple replacements with Arg's were more likely to produce a misfolded inactive protein, but a C-terminal triple mutant had unaltered pore-forming activity. Interestingly, some single Arg mutants (*e.g.*, H257R) showed an increased activity over the WT at moderately acidic pH values, suggesting that positive charge at this position will promote membrane interactions of the T-domain. Neutralizing the charge at the same position (H257Q) resulted in slow leakage. These differences are consistent with our expectations of the role of histidine protonation in the formation of the interfacial intermediate state, a postulated general intermediate of the insertion pathway of non-constitutive membrane proteins.

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