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894-Plat

Resolving Active Ion Transport at the Single Molecule Level for the First Time

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Electrochemical gradients across cellular membranes control a plethora of vital biological processes. These gradients are generated by primary active transporters and are subsequently used to drive the exchange of other solutes through secondary active transporters and to facilitate signaling via ion channels. The macroscopic biological phenomena that channels and transporters give rise to are intimately connected to how they function at the single molecule level. For decades, patch clamp recording has been used to observe the functional dynamics of single ion channels revealing discrete on and off states, subconductance states, and other mechanistically important features that macroscopic experiments cannot probe(*1*). Currently, there aren't any techniques available to investigate transporter function at the single molecule level, thus they are only studied using ensemble biochemical methods.

For a decade we have been developing quantitative fluorescence microscopy based assays of arrayed proteoliposomes for investigating membrane proteins(*2-6*). Here we extended the platform to monitor the single-molecule activity and the regulation of a prototypic P-type ATPase, *Arabidopsis thaliana* H^+-ATPase (AHA2). For the first time we have shown that individual proton pumps are not active continuously but rather transitioning between active and inactive states separated by a large activation barrier ($kJMole^{-1}$). We found that the dynamics of these states form the basis of the regulation of the macroscopic activity either by regulatory R-domain, pH gradients, or ATP. Like for ion channels we often found that regulatory inputs do not affect the *intrinsic* pumping rates but rather active probabilities.

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895-Plat

Protein Interactions that Enable Safe and Efficient Copper Ion Transport in the Human Cytoplasm

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Although copper (Cu) is an essential metal for most living organisms, high levels and free such ions are toxic. In humans, after cellular uptake via the membrane-bound importer Ctr1, Cu is transported to targets by cytoplasmic Cu chaperones: Atox1 delivers Cu to membrane-bound P1B-type ATPases (i.e., ATP7B or Wilson disease protein) in the Golgi (secretory path; here, most Cu-dependent enzymes are loaded with Cu) whereas CCS delivers Cu specifically to cytoplasmic superoxide dismutase. In contrast to bacterial and yeast homologs, ATP7B has six similar metal-binding domains protruding into the cytoplasm: possibly, conformational changes among these regulate overall ATP7B activity. To reveal underlying molecular mechanisms as well as thermodynamic and kinetic driving forces for human Cu transport - from the cell membrane to the Golgi - our strategy involves a range of complementary biophysical experiments on purified proteins, domain constructs and engineered variants. From our studies, we have discovered that (a) the cytoplasmic C-terminus of Ctr1 binds Cu through its HCH motif with a moderate affinity that allows for Cu delivery to Atox1, (b) Atox1 can interact with CCS and exchange Cu implying cross-reactivity between cytoplasmic chaperones, (c) transfer of Cu from Atox1 to metal-binding domains in ATP7B proceeds through Cu-bridged hetero-protein dimers displaying enthalpy-entropy compensation, (d) conformational changes and domain-domain interactions within ATP7B depend on Cu loading status and minute changes in solvent conditions, and (e), in addition to its cytoplasmic chaperone activity, Atox1 may have functionality in the nucleus as it interacted with several DNA-binding proteins in a yeast two-hybrid screen.

896-Plat

Dissecting the Catalytic Cycle of the Serotonin Transporter

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The plasmalemmal serotonin transporter (SERT) regulates serotonin homeostasis and signaling by its reuptake from the extracellular space. In accordance with this prominent role, it is a target of several psychoactive substances, ranging from inhibitors (e.g. cocaine) to substrates (e.g. amphetamines). SERT belongs to the solute carrier 6 family. Hence, it utilizes the electrochemical gradient of co-substrates for the uphill transport of serotonin into the cell. Interestingly, the stoichiometry of this secondary-active transport mechanism has been matter of debate - despite decades of investigation. In addition, the order and kinetics of (co-)substrate binding to SERT have remained enigmatic. Here, we utilized the high temporal resolution of the whole-cell patch-clamp technique to decipher the kinetic determinants of selective ligand recognition, and of (co-)substrate binding and transport - in both, the forward transport and substrate exchange mode of SERT. Based on our electrophysiological data, we provide a comprehensive kinetic model of SERT that accounts for kinetics, stoichiometry, and order of (co-)substrate binding and translocation. We find that, Cl- does not participate in coupling of serotonin transport, but is required for substrate binding. In addition, our data suggest that two Na^+ ions bind to SERT in a sequential order (Na^+-serotonin-Na^+). These findings are incompatible with an electroneutral stoichiometry. Our data shall provide a mechanistic framework for future attempts to integrate functional data with available structural information.

897-Plat

Translocase Activity and Asymmetric Model Membranes Probed by Neutron Scattering

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Biological membranes are almost universally asymmetric with individual leaflets containing different lipid species at varying concentrations. This asymmetry may play a key role in biological functions such as protein-membrane interactions, membrane trafficking, and cellular signaling. Traditional model membrane systems are comprised of symmetric membrane leaflets and though progress has been made in developing asymmetric model membranes, these systems are unstable and easily contaminated. We have developed an actively asymmetric biomembrane model system by purifying and reconstituting the well-characterized *E. coli* phospholipid ABC transporter MsbA into proteoliposomes that mimic the phospholipid composition of a physiological bacterial membrane. Subsequent MsbA-mediated phospholipid translocation and resultant membrane asymmetry was studied using small-angle neutron scattering (SANS). SANS is exceptionally well suited for studying the phospholipid distribution perpendicular to the bilayer plane due to its nondestructive nature and sub-nanometer resolution. Additionally, the hydrogen/deuterium (H/D) neutron scattering contrast variation from isotopic phospholipid labeling enables us to track the translocation of lipid membrane components without introducing bulky fluorescence or spin labels. In this study, the MsbA-mediated translocation of the phospholipid phosphatidylethamolamine (PE) between proteoliposome leaflets and the formation of asymmetric bilayers was studied using SANS for the first time. The sustained asymmetry in these model protocells will be central for future studies on biomimetic membrane systems and their role in diverse membrane-directed biological processes.

898-Plat

Functional Characterization of Calcium-activated Phospholipid Scramblase Activity of nhTMEM16

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