bilayer membrane with extremely high efficiency of over 90 %. When reconstituted an ion transporter, F_oF_1-ATP synthase (F_oF_1), into the bilayers of ALBiC, proton translocation driven by catalysis or membrane voltage were observed, showing that the highly sensitive detection of ion translocation is performed in ALBiC. Next, to explore the feasibility of single-molecule detection of transporter activity, we conducted the same assay in a condition where only a few molecules (0, 1, or 2 molecules) of F_oF_1 were reconstituted into each bilayer. The results showed that the response to proton translocation was no longer homogeneous between chambers, i.e. stochastic and quantized proton translocation was observed, demonstrating that the single molecule analysis of ion translocation catalyzed by transporter protein is first achieved in this study. Thus, the new platform, ALBiC, largely extended the versatility of femtoliter chamber arrays, and holds promise for understanding the working mechanism of transporter proteins as well as for further analytical and pharmacological applications.

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Voltage Dependent Conformational Changes of the Na^+/K^+-ATPase Revealed by Site Directed Fluorometry

Jorge E. Sánchez-Rodríguez[1], Pablo Miranda-Fernández[2], Miguel Holmgren[2], Francisco Bezanilla[1].
[1]Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, USA, [2]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA.

Most of the voltage dependence of the Na^+/K^+-ATPase cycle originates from steps associated with extracellular Na^+ binding/release and occlusion/deocclusion transitions. In order to explore regions of the Na^+/K^+ pump that respond to voltage, we engineered single cysteine mutants facing the outside of the α and β subunits as targets of cysteine-reactive tetramethyl rhodamine (TMRM). These pumps were expressed in *Xenopus* oocytes and voltage-clamped to obtain simultaneous electrical and optical recordings. We have detected voltage dependent fluorescence changes when TMRM is conjugated at sites of the α and β subunits. Interestingly, positions within the β subunit produced robust voltage dependent fluorescence signals. These fluorescence changes follow the kinetics of Na^+ translocation through the pump. Quenching of the fluorescence signal can be altered by tryptophan mutations at the external side of the α subunit, indicating movement of the external face of the α subunit. Surprisingly, in some positions fluorescence changes persist even in the presence of ouabain, a specific inhibitor of the pump, and these changes are modified by tyrosine mutations in the transmembrane segment of the β subunit. These results suggest that there are intrinsic voltage-dependent conformational changes in the Na^+/K^+ pump. Supported by U54GM087519 and GM030376.

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Na^+/K^+-ATPase Pumping Mechanism: Insights from Simulations

Huan Rui, Avisek Das, Benoit Roux.
Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, USA.

The Na^+/K^+-ATPase resides in the plasma membrane and maintains the physiological K^+ and Na^+ concentration gradient across the cell membrane. It functions via a ping-pong mechanism, making iterative transitions between inward-facing (E1) and outward-facing (E2) conformations. The E1 conformation binds three Na^+ from the cytosol and exports them using the energy from ATP hydrolysis. The release of Na^+ and the binding of K^+ at the extracellular side trigger the structural transition to the E2(K2) state, which imports two K^+, followed by the pump returning to the E1 conformation. Although the broad features of the pumping cycle are known, the transition mechanisms between the conformational states and why a given state preferentially binds K^+ or Na^+, two monovalent cations of very similar radius, is not understood. Starting from the available x-ray structures of the Na^+/K^+-ATPase and the SERCA Ca^{2+}-pump, we use anisotropic network model pathway calculations and targeted molecular dynamics simulations to generate atomic models of the outward facing Na^+/K^+-ATPase, Na3E2-P and K2E2-P. Qualitative support of the models comes from a recent Na^+/K^+-ATPase x-ray structure and previous mutagenesis data. In addition to this, the gating charge and dissociation constants of the extracellular Na^+/K^+ binding calculated using the models are in excellent agreement with experimental data as well. The model generation process also produces the occlusion/de-occlusion transition pathways upon ion binding. To study the change in ion selectivity during the occlusion/de-occlusion process, free energy perturbation calculations are performed. The results reveal the molecular determinants of the 3Na-2K stoichiometry of the Na^+/K^+-ATPase.

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Towards Thermodynamic Characterization of Transport Cycle in Secondary Transporters using Enhanced Sampling Techniques

Mahmoud Moradi, Giray Enkavi, Emad Tajkhorshid.
University of Illinois at Urbana-Champaign, Urbana, IL, USA.

Secondary active transporters undergo large-scale conformational changes to couple the uphill and downhill transport of substrates. We have developed a powerful computational approach for the study of secondary active transport by combining several state-of-the-art enhanced sampling techniques primarily based on loosely coupled multiple-copy MD simulations (e.g., Bias-Exchange Umbrella Sampling and String Method with Swarms of Trajectories) within a novel, empirical, iterative sampling framework. Using this novel approach we were able to, for the first time, reconstruct an entire thermodynamic cycle associated with a secondary active transporter, namely, Glycerol-3-phosphate transporter (GlpT), based on its only available crystal structure. We have calculated the free energy profile of GlpT along a ''cyclic'' transition pathway connecting four distinct states of GlpT-phosphate complex including inward-facing apo, inward-facing bound, outward-facing bound, and outward-facing apo. Our results, which are in agreement with alternating access mechanism, indicate that the substrate binding lowers the free energy barrier of the transition between the inward- and ouward-facing states. When the substrate is present, the global conformational changes of the protein are coupled to the substrate translocation within the binding site. These results particularly highlight the significance of coupling between the local conformational changes of the binding site and global conformational changes of the protein. The simulations performed take advantage of tens to hundreds of loosely coupled all-atom MD simulations of GlpT in an explicit membrane/solvent environment with a total simulation time equivalent to ~20 microseconds of single-copy MD on a system of ~125,000 atoms. The novel approach developed here, which attempts to address the complexities associated with large-scale conformational changes of transporters and their coupling with the substrate translocation, may open opportunities for the study of other secondary transporters using enhanced sampling techniques and state-of-the-art supercomputing.

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Protonation States of Key Acidic Residues at the Ion Binding Sites in Na/K Pump by QM Calculations

Naoki Tsunekawa, Chikashi Toyoshima.
The University of Tokyo, Institute of Molecular and Cellular Biosciences, Tokyo, Japan.

Sodium-potassium ATPase transports three Na^+ ions outward and two K^+ ions inward by alternating two major conformational states, E1 and E2. There are three high affinity Na^+ binding sites in E1 and two K^+ binding sites in E2. These binding sites are located closely to each other in a transmembrane region containing several acidic residues. The protonation states of the acidic residues have been postulated to play an important role in ion selectivity. As the protonation states are strongly coupled, pKa estimations with programs, such as MCCE and PROPKA, using empirical point charges are ambiguous. It is questionable if conventional molecular dynamics simulations, though widely used, are applicable to determine the protonation states of strongly interacting residues, especially of protonated carboxyl groups. We therefore introduced quantum mechanical (QM) calculations in order to determine the positions of protons by calculating electrostatic potential (ESP) from electron densities. Starting with a model in which all acidic residues were deprotonated, the ESP showed several minima close to the carboxyl groups. The next model was generated by adding a proton at the minimum, and the ESP was calculated in the same way. Such procedure was repeated several times until no distinct minimum was found near the carboxyl groups. Finally, the geometry of the model was refined by QM to yield the model with explicit protons.

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Na^+/K^+ Pump Ion Binding Site Interactions Regulate the Proton Leak Pathway

Kevin Stanley[1], Craig Gatto[1], Pablo Artigas[2].
[1]School of Biological Sciences, Illinois State University, Normal, IL, USA, [2]Texas Tech University Health Sciences Center, Lubbock, TX, USA.

The Na^+/K^+ pump (NKA) is a membrane bound transporter located in animal cells which transports three Na^+ ions out for two K^+ ions into the cell for every one ATP molecule hydrolyzed. Two out-of-three ion-binding sites within the protein reciprocally bind two K^+ or two Na^+ ions (shared sites), while the remaining site exclusively binds Na^+. Without these two ions present the NKA passively transports H+, likely through the Na-exclusive site. In order to understand the proton transport mechanism voltage-clamp was used to study the