Moreover, we found that under a sufficiently strong torque in the opposite direction of ATP hydrolytic rotations, it rotated in the opposite direction, or the ATP synthetic direction, in a stepwise manner. The torque necessary for rotations in the synthetic direction times 120° was nearly equal to $\in'' \in 1/4$ under various conditions except for conditions at sufficiently low ADP concentrations.

869-Pos

Spatial Distribution of Elasticity in the F1 Motor of ATP Synthase Reveals the Microscopic Nature of the Coupling Between the Central Shaft and the Catalytic Subunit

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F_OF_1-ATPase is a rotary motor protein that synthesize ATP from ADP using the proton gradient across a membrane as a free energy source. The proton flow through the membrane-embedded portion, F_O, is thought to generate the rotary torque that drives the rotation of the asymmetric shaft in the cylinder of hexagonally arranged alpha and beta chains forming the catalytic subunit of the F_1 portion. Mechanical energy of the rotating shaft is used by the active sites of F_1 to synthesize ATP against thermodynamic potential gradient. The microscopic mechanism of this energy conversion is still not fully understood. It was suggested that elastic power transmission with transient storage of energy in some compliant part of the common shaftis required for the high turnover rate to occur [1].

Here we use fully atomistic molecular dynamics simulation to study the spatial distribution of torsional elasticity in the F_1 motor on the 500-ns timescale. The overall range of angular fluctuations of the central shaft with respect to the symmetry axis of the catalytic subunit is consistent with the results of the corresponding experimental study [1]. The detailed analysis of the rotational mobility reveals, however, that the measured range of fluctuations results from two different effects: the internal elasticity of the shaft itself and the effective load imposed on it by the catalytic subunit. Separation of these two effects has led to the detailed description of the dynamic coupling between the shaft and the catalytic subunit. We also propose a simple model of the F_1 motor that might be a useful tool in future studies of the energy transfer in F_OF_1-ATPase.

[1] Sielaff et al. PNAS 105:17760-17765 (2008)

870-Pos

Structure of CopA from Archaeoglobus Fulgidus by Cryoelectron Microscopy

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CopA, a bacterial transporter of Cu^+ and Ag^+ from *Archaeoglobus fulgidus* , was cloned, overexpressed, purified, reconstituted into lipid bilayers and crystallized into tubular crystals in the presence of Cu^+ chelator BCDS by dialysis at 50°C. Three Cryo-EM Structures were obtained with different constructs and lipids 1) dNdC-CopA with DOPC, N and C terminal cytoplasmic peptides truncated of CopA reconstituted into 1,2-dioleoyl-sn-glycero-3-phosphocholine, 2) dC-CopA with DOPC, C terminal cytoplasmic peptide truncated of CopA reconstituted into 1,2-dioleoyl-sn-glycero-3-phosphocholine, 3) dC-CopA with DMPC/DOPE, C terminal cytoplasmic peptide truncated of CopA reconstituted with 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine. All reconstituted proteoliposomes retain their functionality with V_{max} ranging from 1.14 to 2.03 nmol/μg/min and $K_{0.5}$ ranging from 0.1 to 0.8 μM, depending on the reconstituted proteoliposomes and ion substrates. The optimal temperature for enzyme assays of these reconstituted proteoloposomes are located between 65°C to 75°C and these activity measurements were conducted at the temperature of 70°C. Three cryo-EM structures obtained by frozen-hydrated tubular crystals and Fourier processing have resolutions from 12.5 to 17.5 é…. Based on the difference map and the modeling between dC-CopA and dNdC-CopA, N-terminal metal binding domain (MBD) of CopA appears to lie between the ATP binding domain and Actuator domain and has an inhibitory role, which is relieved by receiving Cu^+ from the soluble metals chaperon. Efforts for higher resolution as well as computational modeling of CopA are underway in order to investigate the relative position of cytoplasmic domains with respect to transmembrane helices, in which the transport sites and ion gateway are located.

871-Pos

Sodium Pump A1 And A3 Subunit Isoforms Mediate Distinct Responses To Ouabain And Are Both Essential For Human Neuroblastoma

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The sodium pump (Na^+,K^+-ATPase) maintains the sodium gradient across plasma membranes of animal cells. By hydrolyzing ATP the enzyme transports 3 Na^+ ions out of the cell in exchange for 2 K^+ ions that are taken into the cytosol. This activity can be interrupted by cardiotonic steroids (CTS). Recent publications have, however, established that CTS not only inhibit the sodium pump but that they also induce signalling cascades that influence the physiology of cells in various ways.

Sodium pumps are composed of α and β subunits (and additionally in some tissues of γ subunits) that appear is several isoforms. In some cells different α subunit isoforms are coexpressed, giving rise to the question about the need for their co-existence.

Using human neuroblastoma cells SK-N-AS that co-express α1 and α3 isoforms of the sodium pump α subunit, we selectively silenced either the α1 or α3 subunit by means of small interfering RNA and investigated cell survival and the cellular response to ouabain, a widely used CTS. We find that both of the two α subunit isoforms are essential for cell survival, indicating that substitution of one subunit for the other is not sufficient. In the presence of both α subunits ouabain causes a sustained Erk1/2 activation. This activation is not affected when the α1 subunit is silenced. When α3 expression is silenced, ouabain-induced activation of Erk1/2 does not occur, even at a high concentration of ouabain (1 μM). Thus, ouabain-induced Erk1/2 activation is mediated in SK-N-AS cells by α3 only, and α1 does not participate in this event. This is the first demonstration of selective involvement of a specific sodium pump α subunit isoform in ouabain-induced signaling.

872-Pos

Ion-Selectivity of Externally Facing Na^+-Exclusive and Na^+/K^+-Shared Sites in the Na/K-Pump

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The Na/K-pump extrudes 3 Na^+ in exchange for 2 K^+ across the plasmalemma of animal cells. Two-out-of-three ion binding sites in the protein can be occupied by either Na^+ or K^+, whereas another site exclusively binds Na^+. At maximally activating [K^+_o], Na^+_o binding to the Na^+-exclusive site (first site to open in sequential Na^+ release) is manifested as [Na^+_o]- and voltage-dependent inhibition of outwardly-directed (due to the 3:2 stoichoimetry) pump current (I_P). Guanidinium$^+$ can also backward-transit this Na^+-release channel inhibiting I_P at negative voltages (Yaragatupalli et al. (2009) PNAS **106**:15107-15512). To study the ion-selectivity of this Na^+-release channel we measured voltage-dependent inhibition of I_P with external solutions containing different cations (120-125 mM). This inhibition followed the sequence: $Na^+_o > Li^+_o >$ guanidinium^+_o >aminoguanidinium^+_o > acetamidinium^+_o ~diaminoguanidinium^+_o ~formamidinium^+_o ~Cs^+_o ~K^+_o ~N-Methyl-D-Glucamine (NMG^+_o).

Ouabain-sensitive currents in the absence of Na^+_o and K^+_o were also measured. An inward current (possibly representing leakage through the Na-exclusive site when the shared sites are empty) was observed in NMG^+_o, guanidinium^+_o and aminoguanidinium^+_o. The other cations tested induced ouabain-sensitive outward currents at all voltages. Ouabain-sensitive current amplitude in 120 mM acetamidinium^+_o was similar to maximal K^+_o-induced I_P. Without Na^+_o, [acetamidinium ^+_o] of outwardly-directed current gave $K_{0.5acet+}$ ~10 mM indicating that this ion acts as a low-affinity K^+ surrogate. Consistently, in sheep kidney purified enzyme preparations, both acetamidinium$^+$ and formamidinium$^+$ induced ouabain-dependent ATPase activity ($K_{0.5acet}$ = 80 mM, $K_{0.5form}$ = 113 mM, $K_{0.5K+}$ = 0.9 mM). Transport was confirmed by means of ouabain-sensitive C^{14}-acetamidinium uptake. Our results indicate that acetamidinium$^+$ and formamidinium$^+$ can be transported like K^+ by the Na/K pump. Molecular dynamics simulations based on an atomic model are used to explain organic cation coordination in the occluded form. Supported by NIH DK083859 and GM062342.

873-Pos

The Route and Mechanism of Uncoupled Current Flow through Na/K-ATPase Pumps Lacking the Two COOH-Terminal Tyrosines

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Na/K-ATPase pumps generate outward current during ATP-driven stoichiometric exchange of three intracellular Na ions for two extracellular K ions. At acidic pH, in the absence of extracellular Na and K ions, an uncoupled current flows through wild-type Na/K pumps at large negative membrane potentials, believed carried by protons. Both currents are abolished by the specific Na/K pump inhibitor ouabain. In *Xenopus*α1 pumps made less sensitive to ouabain by mutations Q120R/N131D or C113Y we observed a similar uncoupled current in the absence of extracellular Na and K ions even at physiological pH

(7.6). After deletion of the last five (∈″KESYY), or two (∈″YY), COOH-terminal residues of those pumps, uncoupled current was recorded also in extracellular Na. We used two-microelectrode recording to measure uncoupled, and coupled Na/K transport, currents as those inhibited by 10-30 mM ouabain in *Xenopus* oocytes expressing C113Y or C113Y-∈″YY Na/K pumps. To investigate whether the uncoupled current traverses the same principal pathway followed by transported Na and K ions we used two methods to close that pathway. In one method, we formed stable BeF_X-Na/K-pump complexes, trapped in an E2P-like state with closed cytoplasmic-side gates, by injecting oocytes with 1mM BeF_X. In the other, we closed the extracellular access pathway by modifying a Cys substituted for T806, at the outer end of TM6, with 1 mM extracellular MTSET. Both methods abolished coupled Na/K pump transport current, as well as Na current flow through palytoxin-bound C113Y Na/K pump-channels. But neither method diminished uncoupled current at −180 mV, suggesting either that the responsible ions do not traverse the principal pathway shared by transported Na and K ions or, if they do follow that route, that they do not travel as hydrated cations. [NIH HL36783]

874-Pos

Intracellular Proton Binding is Voltage-Dependent and Rate-Limiting for the Gastric H,K-ATPase Under in vivo Conditions

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Compared to the extensively studied Na,K-ATPase reaction cycle, much less is known about the voltage-dependent steps in the pump cycle of gastric H,K-ATPase. Due to the overall electroneutral transport stoichiometry of the proton pump, its voltage-dependent ion transport properties cannot readily be determined by standard electrophysiological techniques.

Therefore, we use a combination of two biophysical techniques to investigate H,K-ATPase activity: voltage-clamp-fluorometry on the tetramethylrhodamine-6-maleimide-labeled H,K-ATPase variant αS806C to monitor the voltage-dependent distribution between E_1P/E_2P-states (Ref. 1) and voltage-controlled Rb^+ uptake measurements to assess the steady-state ion transport activity under various pH and ionic conditions in *Xenopus* oocytes.

Both the steady-state E_1P/E_2P-distribution and Rb^+ uptake of the gastric H,K-ATPase are highly sensitive towards changes in intracellular pH (which can be achieved by adding weak organic acids like butyric acid to the extracellular solution), whereas even larger changes in the extracellular pH do neither influence the conformational E_1P/E_2P-equilibrium nor transport activity. An intracellular acidification of approximately 0.5 pH units results in a large negative shift (~100 mV) of the voltage-dependent fluorescence amplitudes and an approximately two-fold acceleration of the reciprocal time constants at positive membrane potentials.

One possible interpretation of these results is that proton binding takes place in a shallow intracellular ion access channel (apparent well depth: 0.3-0.5).

Since maximal rubidium uptake at saturating concentrations is strongly stimulated by intracellular acidification, the voltage-sensitive intracellular proton binding step is apparently rate-limiting for the overall transport activity under physiological conditions. These findings highlight the need for cellullar mechanisms which increase the availability of protons at the cytoplasmic face of the pump, such as CO_2-producing mitochondria, sub-membrane carbonic anhydrase and the basolateral Cl^-/HCO_3^- exchanger that are characteristic for parietal cells.

References
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875-Pos

Conformational Dynamics of a Fluorescent Probe Attached to the Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA) Studied by Molecular Simulations

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We have performed molecular dynamics simulations, based on a new X-ray crystal structure of fluorescent-labeled SERCA, in order to establish a more rigorous foundation for analyzing fluorescence data from this system. Site-specific labeling of a protein with a fluorescent probe can provide insight into local structural dynamics, based on fluorescence quenching or anisotropy measurements, or based on fluorescence resonance energy transfer (FRET) to another label. SERCA was labeled at position Cys674 in the P-domain with the fluorescent probe IAEDANS. The crystal structure of IAEDANS-labeled SERCA was determined to 3.4Å resolution, which was sufficient to show the IAEDANS label in close proximity to residues Arg615 and Arg620. This structure was used as a starting point for molecular dynamics simulations and conformational sampling calculations of the fluorescent probe and its protein environment. To be able to perform these simulations, we developed CHARMM force-field parameters for the fluorescence probe IAEDANS. Quantum chemistry calculations have also been performed on the ground state and excited states of IAEDANS, to determine the orientation of the transition dipole moment. The transition dipole autocorrelation functions and reorientation times were calculated from the simulated trajectories and compared with experimental measurements by fluorescence anisotropy. These results validate our computational approach and establish a reliable framework for analysis of fluorescence experiments in this system. This work was supported by NIH (GM27906, AR007612) and the Minnesota Supercomputing Institute.

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Measuring the Dissociation Constants of Ligands from PMCA Complexes by a Photoactivatable Phosphatidylcholine Membrane Domain Probe

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The purpose of this work was to obtain structural information about conformational changes of the plasma membrane Ca^{2+} pump (PMCA) in the membrane region upon interaction with ATP, Ca^{2+}, calmodulin and acidic phospholipids. To this end, we have quantified labeling of PMCA with the photoactivatable phosphatidylcholine analog [^{125}I]TID-PC/16, measuring the shift of conformation E_2 to the auto-inhibited conformation E_1I and to the activated E_1A state, titrating the effect of Ca^{2+} and ATP under different conditions. With this method we were able to measure apparent and equilibrium constants for the dissociation of Ca^{2+}, ATP and calmodulin and other ligands from PMCA complexes through the change of transmembrane conformations of the pump. The results indicate that the PMCA possesses a high-affinity site for Ca^{2+} regardless of the presence or absence of activators. Modulation of pump activity is exerted through the C-terminal domain, which induces an apparent auto-inhibited conformation for Ca^{2+} transport but does not modify the affinity for Ca^{2+} at the transmembrane domain. The C-terminal domain is affected by calmodulin and calmodulin-like treatments driving the auto-inhibited conformation E_1I to the activated E_1A conformation and thus modulating the transport of Ca^{2+}. The data further suggest that the hydrophobic transmembrane domain of the PMCA undergoes major rearrangements resulting in altered lipid accessibility upon Ca^{2+} binding and activation. With grants from ANPCYT, CONICET, UBACYT and NIH.

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A Phospholamban-Cardiac Ca^{2+} Pump Fusion Protein Retains Full Functional Regulation

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To study the molecular mechanism by which phospholamban (PLB) inhibits the cardiac Ca^{2+} pump (SERCA2a), we engineered a fusion protein with PLB tethered to the C-terminus of SERCA2a (SER-20G-PLB). A 20 glycine-residue linker was inserted between the C-terminus of SERCA2a and the N-terminus of PLB to allow the PLB-tether either to bind to SERCA2a at its inhibition site, or to diffuse away from SERCA2a in the membrane, permitting enzyme activation. SER-20G-PLB was expressed in insect cell microsomes and compared to normal WT-SERCA2a co-expressed with normal WT-PLB. SER-20G-PLB ran at 110 KDa and 550 KDa on SDS-PAGE, demonstrating that the fusion protein exists as both monomers and homo-pentamers, like WT-PLB which ran at 5 KDa and 25 KDa. In the Ca^{2+}-uptake assay, SER-20G-PLB transported Ca^{2+}, showing that the pump remained fully active. Importantly, the Ca^{2+}-uptake by SER-20G-PLB was stimulated by the anti-PLB antibody, 2D12, in a similar fashion as occurred with WT-SERCA2a co-expressed with WT-PLB. Further, in the Ca^{2+}-dependent ATPase assay, the Ca^{2+} concentration for half-maximal activation (K_{Ca} value) was 0.26 ± 0.01 μM for SER-20G-PLB, identical to 0.25 ± 0.01 μM for WT-SERCA2a co-expressed with WT-PLB, both larger than 0.16 ± 0.01 μM for WT-SERCA2a expressed alone. Thus, SER-20G-PLB has intrinsically decreased apparent Ca^{2+} affinity, the hallmark of PLB inhibition. Finally, the L31A mutation in the PLB-tether, which disables normal PLB function, also prevented Ca^{2+}-ATPase inhibition by the tether (K_{Ca} = 0.16 ± 0.01 μM). Thus, SER-20G-PLB retains a fully active Ca^{2+} pump, which is intrinsically regulated by its flexibly anchored PLB-tether. The fusion protein, with a built-in 1:1 molar stoichiometry between PLB and SERCA2a, provides a unique system to address dynamic interactions between the two proteins situated in the membrane.