Recombinant Production of Human Aquaporin-1 to an Exceptional High Membrane Density in Saccharomyces Cerevisiae

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porin-OmpPst1 of Providencia stuartii is investigated in its susceptibility for two carbapenem antibiotics imipenem and meropenem. Electrophoresis measurements coupled to kinetic parameters of antibiotic binding to the channel. Activity of antibiotics against bacteria determined by microbiological assays correlates with the results obtained from liposome swelling assay and planar lipid bilayer measurements. In addition, the effect of divergent and trivalent cations on antibiotic affinity to the channel is investigated. To follow the exact translocation pathway, molecular modeling provides atomistic details on the interaction of specific side chains of the two antibiotics with the channel residues and the position of affinity sites. Further, mutation of these specific sites in the channel and recording ion current fluctuation in presence of antibiotics reveal the rate limiting interaction for its translocation. Here, we have concluded on the relation between permeation of the antibiotics and resistance acquired by Providencia stuartii. Moreover, employing a multidisciplinary approach from MD simulations to protein engineering helps to get a picture of the system. This information further might give insights for rational drug design, for effective uptake of antibiotics through porins- check point of the cell.

References:

2816-Pos Board B508
Antibiotic Transport through Porins
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The emergence and dissemination of coordinated resistance mechanism (MDR) underline the importance to understand the rate limiting steps in antibiotic action. Here OmpF porin from E. coli is used as an example to demonstrate what limits the penetration of carbapenems. Bacteriological kinetic killing assays, temperature dependent ion conductance measurements, and all-atom computer simulations were combined to study interaction and translocation of clinically relevant β-lactam antibiotics through wild type OmpF and two mutants D113A and D121A, where the key residues at the constriction region have been substituted. Expression of these various OmpF mutants in an otherwise porin-null bacterial strain revealed an increase of bacterial susceptibility for the mutants. High-resolution conductance measurements and modulating the temperature indicates lower energy barriers for mutant porin correlating with the microbiological assays. All atom modeling provided a most probable pathway able to identify the relevant side-chains interactions. This combined approach allows identifying rate limiting interaction and suggests possible modification to enhance antibiotic penetration.

References:

2817-Pos Board B509
Characterization of a Cyanobacterial Outer Membrane Protein: An E. Coli Tolc Homologue from Synechocystis Sp. Pcc 6803
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E. coli Tolc (tolerance to colicins) represents an interesting class of outer membrane (OM) proteins, as it has an α-helical periplasmic tunnel and β-barrel membrane region, providing a conduit for export of metabolites and xenobiotics from cell interior to exterior, and import of colicin E1 (1). A TolC homologue, Slr1270 from Synechocystis 6803, cloned and expressed in E. coli, has > 40% similarity and ~16% identity to E. coli and Pseudomonas counterparts, and has a similar domain organization. Homology modeling using Pseudomonas OpmM as template modelled 93% of Slr1270 sequence. The 1581bp slr1270 gene was cloned and overexpressed in E. coli. Protein from inclusion bodies, refolded through step-wise dialysis showed major bands at ~55kDa and >150 kDa on SDS-PAGE corresponding to the monomer and trimer respectively and ~300 kDa on CN-PAGE. Purified protein displays a far-UV CD spectrum characteristic of E. coli TolC with > 50% α-helix, and formed channels in planar lipid bilayers with a characteristic single channel conductance of ~50 pS in 0.1M NaCl. The intact protein mass spectrum (LC-MS) with a major peak at 54,489 probably represents a mixture of two species, the TolC product with 1-40 removed and an intact 6-His tag (calculated mass 54,457.1 Da), and a product with 1-38 removed and a 5-His tag (calculated mass 54,490.2 Da) after a single carbamoylapiase event. The small peak at 54,638 Da probably corresponds to TolC product with 1-38 removed and an intact 6-His tag (calculated mass 54,627.3 Da). Peptides 39-76 and 41-76 were recovered from trypsin digests confirming N-terminal hexa-histidine tag (1) Zakharov, S. D. et al. 2012. Pathways of Colicin Import: Utilization of BtuF, OmpF Porin, and the TolC Drug Export Protein, Biochem. Soc. Trans., 40, 1463-1468.

2818-Pos Board B510
Electrophysiological Analysis of PapC Mutants Provides Insights into the Mechanism of Plug Displacement
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The PapC usher is a twin beta barrel pore of the outer membrane of urapathogenic E. coli used for the assembly of the P pilus, a key virulence factor in bacterial colonization of human kidney cells. The usher catalyzes the translocation and ordered addition of folded pilus subunits delivered by the chaperone PapD to the growing pilus. Each PapC monomer is a 24-stranded beta barrel, flanked by N- and C-terminal globular domains and occluded by a large plug domain (PD). The C-terminal helix and the beta 5-6 hairpin loop are additional structural components that may play a role in controlling plug dynamics. Indeed, structural studies have revealed that the PD is released to the periplasmic side during pilus biogenesis, but the exact mechanism for PD displacement has not yet been elucidated. Several key residues that may be critical for plug stabilization have been proposed. They cluster in regions at the interface of the plug, the barrel, the alpha helix and the hairpin, and appear to belong to either electrostatic or allosteric networks. In order to assess the roles of these residues in plug displacement, we have used planar lipid bilayer and patch-clamp electrophysiology and compare the activity of the wildtype channel with that of mutant channels with either single or multiple alanine substitutions at these sites. Many mutants showed an increased propensity at plug displacement, as witnessed by openings with a conductance similar to that of the previously characterized plugless mutant (~3 nS). Others displayed quieter than WT activity. In addition, evidence of modular gating was observed in WT and some mutants. Together, these mutants provide insight into the molecular mechanism of PD displacement for pilus assembly and translocation through the PapC usher.

2819-Pos Board B511
Recombinant Production of Human Aquaporin-1 to an Exceptional High Membrane Density in Saccharomyces Cerevisiae
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Due to the lipid nature of cellular membranes preventing transport of most solutes between the cytosol and the extracellular environment as well as from the cytosol to the interior of organelles, cellular homeostasis relies on integral membrane proteins allowing selective trans membrane movement of solutes. Aquaporins constitute a family of physiologically very important integral membrane proteins that are found in all three kingdoms, eubacteria, archaea and eukaryotes. As protein channels, they facilitate passive transport of water and small solutes such as gases and organic compounds. Aquaporins are mainly composed of two membrane spanning domains. Aquaporin CDNA was expressed from a galactose inducible promoter situated in the genomic DNA of Saccharomyces cerevisiae. Due to the lack of native machinery to transport proteins across the yeast plasma membrane, heterologous expression was achieved in yeast cells transformed with a plasmid containing the Gene of interest. The expression was confirmed by Western blotting and Coomassie staining of cell lysates. After expression at 15°C in a yeast host over producing the Gal4p transcription factor and growth in amino acid supplemented minimal medium. In gel fluorescence with western blotting showed that low accumulation of correctly folded recombinant Aquaporin-1 at 30°C was due to in vivo mal-folding. Reduction of the expression temperature to 15°C almost completely
predicted Aquaporin-I mal-folding. Bioimaging of live yeast cells revealed that recombiant Aquaporin-I accumulated in the yeast plasma membrane. A detergent screen for solubilization revealed that CymA-L5 was superior in solubilizing recombinant Aquaporin-I and generated a monodisperse protein preparation. A single Ni-affinity chromatography step was used to obtain almost pure Aquaporin-I.

**2820-Pos Board B512**
Aquaporin Trafficking as a Specific Regulatory Mechanism to Adjust Membrane Water Permeability


Plant aquaporins regulate water fluxes across membranes by enhancing membrane water permeability (P). In particular, the plant plasma membrane holds PIPs, one of the largest groups of aquaporins. PIPs are divided in two clusters (PIP1 and PIP2) that disclose intriguing aspects: i) the potential of modulating P, by PIP1-PIP2 co-expression, distinguished for each PIP showing differential capacity to reach the PM and ii), the factor to reduce water permeation through the pore after cytosolic acidification, as a consequence of a gating process. Our working hypothesis is that cytosolic pH (gating) and PIP co-expression (trafficking) enhance plasticity to the membrane water transport capacity as a consequence of a PIP1-PIP2 cooperative interaction. Thus, PIP1 cellular trafficking and its effect in water permeability emerge as playing a key role as a regulatory mechanism. To analyze this interaction we used PIP1-PIP2 pairs from different species (*Fragaria ananassa* and *Beta vulgaris*). Our experimental approach included i) designing mutants to alter the PIP1-PIP2 interaction by means of site directed mutagenesis; ii) tracking aquaporin localization –at internal structures or expressed at the level of the PM-; and iii) analyzing water transport capacity in control and inhibited (medium acidification) conditions by means of measuring P, in Xenopus oocytes. Our finding support evidences in agreement with the concept that PIP2 and PIP1 interact to form functional heterooligomeric assemblies, and thus the composition of these PIP assemblies determines their functional properties. As PIP1 alone is not able to reach the plasma membrane its contribution to enhance water permeability is associated to its translocation and interaction with a PIP2. This regulatory mechanism seems to be present in different vascular plants. This information is integrated in a proposal for water transport pathways including the organs where these PIPs are present.

**2821-Pos Board B513**
Selectivity Filter Scanning of the Human Voltage Gated Proton Channel Hv1

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The human voltage-gated proton channel Hv1 is electrogenic, and the C1M channel resembles WT in selectivity, kinetics, and specific conduction was restored with Asp or Glu at position 116. The D112V/N116D channel (TVGDG) is similar to that of the K-selective KcsA channel (TVGYG), yet NaK conducts both Naþ and Kþ equally well. To investigate the non-selectivity mechanism of NaK, we performed non-equilibrium molecular dynamics simulations using step-wise pulling protocols and Jarzynski’s Equality. Two ions in the crystal structure of NaK were kept at site S3 (z = 14.0 Å) in the filter and in the small vestibule above S3, and one ion for pulling was placed at z = 0 Å below the filter entrance. Two sets of simulations with either NaCl or KCl were compared to quantify how the filter dehydrates and conducts the different ions along the z-axis toward the extracellular surface. The simulations show that the entry of Naþ into the filter is favored by about 0.5-2 kcal/mol over Kþ due to easier dehydration of Naþ. This difference is attributed to the collapse of S3 by 11% in volume on Naþ, resulting in a higher negative charge density of carbonyl oxygen atoms around Naþ than Kþ. When ions enter the filter and displace the ion at S3 (called Na3 or K3) into the small vestibule, the pulled Kþ in S4 (below S3) is more dehydrated than the pulled Naþ. K3 and the other Kþ and water molecules in the filter become significantly more symmetric around the z-axis than Naþ and water molecules. This symmetric distribution of Kþ ions and water favors the movement of Kþ above S3, offsetting the slight ion selectivity at the filter entrance. We hypothesize that the non-selectivity filter of the NaK channel favors Naþ over Kþ below S3, but becomes more selective for Kþ than Naþ above S3, thus resulting into the non-selectivity as experimentally observed.

**2824-Pos Board B516**
Statistics of Simulated Ion Channels

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The statistical analysis of ionic currents through biological ion channels is straightforward when single channels are recorded. However, analysis can be intractable when more than one channel is present due to signal overlap. We present a statistical analysis of simulated ion channel recordings when the number of channels is small. Through numerical analysis, we display relationships...