

1934-Pos**Investigation of conformational sampling of LeuT due to Na site mutations at thr 354 and ser 355 with DEER EPR spectroscopy**Brynn E. Elson¹, Kelli N. Kazmier², Richard A. Stein³.

¹Biochemistry, Hillsdale College, Hillsdale, MI, USA, ²Chemistry, Hillsdale College, Hillsdale, MI, USA, ³Vanderbilt University, Nashville, TN, USA. LeuT, an integral membrane protein and member of the NSS family, is a bacterial homolog of human neurotransmitter transporters. The mechanism of LeuT transport has been described by three conformations captured by X-ray crystallography: outward-facing, outward-facing occluded, and inward-facing. To select for an inward-facing conformation, a number of conserved residues were mutated. Using DEER EPR spectroscopy, we have investigated the effects of one set of these mutations, T354V and S355A, in LeuT. In this data, we describe the effects of the loss of Na binding on the conformational sampling as well as evaluate whether the crystallographic inward-facing structures represents a natively-sampled LeuT conformation.

1935-Pos**The molecular puzzle of the human serotonin transporter: Where a proton and a potassium fit in the picture**Eva Hellsberg¹, Danila Boytsov², Walter Sandtner², Lucy R. Forrest¹.

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The human serotonin transporter hSERT, or SLC6A4, from the solute carrier 6 (SLC6) protein family, plays an important role in the central and peripheral nervous systems. Its major neurobiological task is the termination of serotonergic neurotransmission by reuptake of its endogenous substrate, serotonin, into pre-synaptic boutons after signaling. The transporter undergoes several conformational changes in each physiological substrate uptake cycle and interacts with protons, sodium, chloride, and potassium ions during these transitions. Over the years, a substantial body of knowledge about hSERT function and mechanism has been accumulated, significantly improving pharmacological treatment options especially for major depression by covering several chemical compound classes. Nevertheless, effective symptom amelioration, side effect profiles, and patient compliance are still far from ideal and require urgent action. For this reason, a fundamental approach is to tackle key unanswered questions regarding the transporter's function, including the mechanism of potassium and proton binding, whose roles are both interconnected and poorly understood. To this end, we have applied molecular simulation approaches at the coarse-grained and fully-atomistic levels, including free energy calculations. The simulations predict the locations of the potassium and proton binding sites and suggest the molecular origins of selectivity at each site. In addition to the gains in basic scientific understanding, the knowledge obtained paves the way for designing mechanism-based transporter ligands that are useful as highly specialized therapeutic agents.

1936-Pos**Investigation of the transport cycle of sodium glucose transport proteins through computational simulation**Fiona B. Naughton¹, John M. Rosenberg², Michael Grabe³.

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The human Sodium-Glucose Linked Transporter hSGLT2 is responsible for the reabsorption of sugar in the kidneys, and as such is a key target for the treatment of type 2 diabetes. SGLTs are found in the cell membrane and use the sodium gradient to transport sugar into cells through an alternating-access mechanism. Experimentally derived structures of the bacterial homolog vSGLT in an inward-open conformation (apo and substrate bound) and the related sialic acid transporter in an outward-open conformation, along with more recent structures of partially inward-open hSGLT1 and partially outward-open inhibitor-bound hSGLT2, give some insight to the transport mechanism. However, the exact nature of the conformational transition and the energetic details that govern the transport cycle remain unclear. We have used molecular dynamics simulations to explore the dynamics of SGLT in the presence or absence of substrate, including unbiased simulations in different conformations, and weighted ensemble simulations using WESTPA to explore the conformational transition landscape. These simulations demonstrate how the presence of substrate can alter the energetics of the conformational landscape; for example, how sugar may lock the outer gate closed, preventing substrate leaks. Improving our understanding of SGLT function through this work is a key step to better inform the treatment of diabetes and other SGLT-related diseases.

1937-Pos**An electrophysiological approach for low turnover and intracellular transport proteins: Improving amplification and accessibility**George Okeyo¹, Andre Bazzone², Maria Barthmes², Rocco Zerlotti², Cecilia George², Niels Fertig².

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SSM-based electrophysiology is an emerging technique extending the range of applications in electrophysiology. The method allows adsorption of any membranes, native, cell culture derived or organellar, to a lipid coated sensor (solid supported membrane, SSM). The large sensor of 3-mm diameter generates a 1000-fold or higher amplification compared to conventional patch clamp. These two features render the method ideal for electrogenic transporters and channels which are difficult to access by other electrophysiological methods due to their low current amplitude or because of their expression in intracellular membranes. Here we present case studies from different protein target classes demonstrating the properties of the method: Transport and binding signals of electrogenic sugar transporters (SGLT, Xyle) and kinetic parameters like rate constants, KD, KM were resolved, pharmacological properties of inhibitors were tested in PepT1 and EAAT2, and the suitability for intracellular membranes was demonstrated by activity measurements from TMEM175 in lysosomes.

1938-Pos**Cryo-EM structure determination of the human reduced folate carrier SLC19A1 in complex with methotrexate**Nicholas J. Wright¹, Justin G. Fedor¹, Han Zhang^{2,3}, Pyeonghwa Jeong⁴, Yang Suo¹, Jiho Yoo^{1,5}, Jiyong Hong⁴, Wonpil Im^{2,3}, Seok-Yong Lee¹.

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Folates are important nutrients that play critical roles in cellular metabolism. Owing to the fact mammals lack systems for folate biogenesis, cells rely on folate transporters and receptors for membrane translocation of these organic anions. The human reduced folate carrier (hRFC), member of the solute carrier 19 family (SLC19), is the main transporter of dietary and reduced folates in humans. Further, hRFC is an anion exchanger that is also responsible for cellular entry of antifolate chemotherapeutics such as methotrexate and pemetrexed. This is exemplified by the fact genetic variants in hRFC or the expression level of hRFC have been implicated in methotrexate resistant cancers. Despite the importance to human metabolism and pharmacology, little was known about the molecular basis of antifolate drug recognition by hRFC. As hRFC is a small solute carrier that was refractory to crystallization, we turned to cryo-electron microscopy for structure determination. An unanticipated limitation encountered was absence of stable methotrexate binding to detergent-purified transporter. Utilizing an engineered fiducial, a methotrexate analog that covalently modifies the substrate binding site, and *ad hoc* image processing strategies, we were successful in solving a cryo-EM structure of hRFC trapped in a methotrexate bound state. In combination with transport assays and molecular dynamics simulations, our studies uncover the molecular basis of methotrexate recognition and other key features of anion selectivity by hRFC. These findings are discussed in depth in our companion poster ["Methotrexate recognition by the human reduced folate carrier SLC19A1"].

1939-Pos**Methotrexate recognition by the human reduced folate carrier SLC19A1**Justin G. Fedor¹, Nicholas J. Wright¹, Han Zhang^{2,3,4}, Pyeonghwa Jeong⁵, Yang Suo¹, Jiho Yoo^{1,6}, Jiyong Hong⁵, Wonpil Im^{2,3,4}, Seok-Yong Lee¹.

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The human Reduced Folate Carrier (hRFC) is essential for folate uptake into mammalian cells. As a critical substrate in nucleotide biosynthesis and amino acid metabolism, folate is required for cell division. Consequently, cancerous cells rely heavily upon folate for their growth, so specific targeting by antifolate pharmaceuticals is highly sought after. One such anti-folate chemotherapeutic is methotrexate (MTX), which requires hRFC for uptake into the cell and variants of which can lead to drug resistance and folate deficiency. Unravelling the transport mechanism of hRFC, as well as the basis for its interaction with folates and anti-folate drugs has important implications for chemotherapeutic development and personalized medicine. In our companion poster ["Cryo-EM structure determination of the human reduced folate carrier

SLC19A1 in complex with methotrexate"] we detail the biochemical and structural techniques required for elucidating the structure of MTX-bound hRFC (65 kDa). Here we present transport assay data and MD simulations that build upon the structure to identify the features of hRFC that make it capable of transporting a wide range of anions, while also probing the molecular determinants of substrate specificity. This multifaceted study provides not only insight into the transport mechanism of hRFC but also a basis for rational chemotherapeutic drug design.

1940-Pos

Residues involved in proton-coupled transport of antimalarial drugs by PfCRT

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A major setback in the fight against malaria has been the emergence of *P. falciparum* parasites resistant to chloroquine (CQ), the primary antimalarial drug. Neutral CQ enters the parasite's digestive vacuole (DV) and obtains two protons preventing its passive efflux through the membrane. The trapped CQ²⁺ kills the parasite by disrupting the detoxification process of heme released from proteolyzed host hemoglobin. CQ-resistant parasites show mutations in a DV membrane transporter, *P. falciparum* CQ resistance transporter (PfCRT), that enables active, proton-coupled efflux of CQ²⁺. While biochemical measurements established the coupling of PfCRT's transport function to the DV membrane proton gradient, the proton-coupled transport mechanism has remained elusive. Particularly it is still unclear what the proton binding site(s) is(are). Recently, the cryo-EM structure of PfCRT from a CQ-resistant variant was determined with a bound Fab fragment occupying its binding pocket. Using us-long all-atom molecular dynamics (MD) simulations we examined the stability of the transporter in the DV-open conformation in the absence of bound Fab fragments, discovering surprising protein stability upon removal of Fab. Next, utilizing constant pH (cpHMD) simulations at 7 different pH values, and considering Galvani potential offset corrections, we determined the pKa of the four titratable residues (H97, D137, D326, and D329) lining the binding pocket. These simulations allowed unambiguous determination of the primary site responding to DV's acidic environment. Finally, with protonation states compatible with the acidic pH of the DV for the aforementioned residues, we studied binding of CQ²⁺ to the transporter via a series of driven MD simulations. We then repeated the cpHMD simulations and determined the proton binding site in the presence of the bound CQ²⁺. Our results shed light on PfCRT's transport mechanism and open new possibilities to design new effective drugs against malaria.

1941-Pos

Phylogenetic and localisation analyses of mammalian VNUT reveal a unique ER-retention sequence replaces vesicular targeting motifs in the C-terminus

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Adenosine 5'-triphosphate (ATP) acts as a neurotransmitter in central and peripheral nerves, and the Vesicular Nucleotide Transporter (VNUT) is thought to load synaptic vesicles (SVs) with ATP. Related neurotransmitter transporters use C-terminal dileucine and tyrosine motifs to target them to SVs. We previously found that (1) rat, mouse and human VNUT weakly localise to synaptic vesicle pools, and (2) that their C-terminals contain a KDEL-like endoplasmic reticulum retention motif rather than a SV-targeting motif. Here we report a phylogenetic analysis of VNUT's C-terminal KDEL-like motif and study whether it promotes endoplasmic reticulum/Golgi retention of VNUT.

We identified 564 VNUT peptide sequences (>300 amino acids long) in NCBI's Nucleotide database. We performed regex analyses to identify KDEL-like ((K/R/H)(D/E)(D/E/L), tyrosine (YxxΦ) and dileucine ((D/E)x{2,3}ΦΦ) motifs, which were mapped to an evolutionary tree using time-tree.org and visualised using EMBL's Interactive Tree of Life. The KDEL-like C-terminal motif is unique to mammals, and transitions from tyrosine and/or dileucine motifs, found in chordate lineage, both older and younger than mammals, at marsupials and monotremes. We studied subcellular localisation for EGFP-VNUT(rat) in Neuro2A and A7r5 cells. Deletion of the KDEL-like motif decreased EGFP-VNUT co-localisation with endoplasmic reticulum-targeted BFP, while maintaining Golgi-DsRed colocalization. Studies with VNUT (rat) -pHluorin revealed that VNUT-pHluorin localizes to mildly acidic

(pH 6.5-7.0) compartments rather than acidic (pH<5.5) compartments like SVs or lysosomes.

In conclusion, the C-terminal motif of mammal VNUT is unique among neurotransmitter transporters like VMAT2, VACHT or VGLUT1-3, and VNUT does not appear to localize to acidic vesicles. This suggests that the loading vesicles with ATP in mammals may occur by non-canonical mechanisms, such as loading at the Golgi apparatus, which would constitute a novel aspect of purinergic signalling.

1942-Pos

Dual transport function of uncoupling protein 3

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The mitochondrial membrane uncoupling protein UCP3 belongs to the SLC25 solute carrier family and transports protons across the inner mitochondrial membrane. Based on the knowledge that another uncoupling protein - UCP2 - transports C4 metabolites (malate, oxaloacetate, and aspartate) and there is high homology between UCP2 and UCP3, we hypothesized that UCP3 also transports these metabolites. To test this, we measured the transport of C4 metabolites against phosphate (³²Pi) in proteoliposomes reconstituted with recombinant murine UCP3. We found that UCP3 transports primarily aspartate and sulfate, but also malate, malonate, oxaloacetate, and succinate. The transport rates from the exchange of ³²Pi for extraliposomal aspartate and sulfate were 0.89 ± 0.33 and 0.61 ± 0.12 μmol/min/mg, respectively. We demonstrated that UCP3 preferentially transports malate, oxaloacetate, and aspartate against phosphate plus a proton from opposite side of the membrane, which does not exactly match the transport pattern of UCP2. Because UCP3 and UCP2 are expressed in different tissues², we anticipate that their substrate specificity may differ. The putative physiological role of UCP3-mediated substrate transport in cells dependent on fatty acid oxidation is discussed. 1. Vozza A, et al. UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. *Proc. Natl. Acad. Sci. U S A* 111, 960-965 (2014). 2. Pohl EE, Rupprecht A, Macher G, Hilde KE. Important Trends in UCP3 Investigation. *Front Physiol.* 10, 470 (2019).

1943-Pos

Conformational and functional interrelation between mitochondrial carrier proteins: UCP4, ANT, P_iT

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Mitochondrial uncoupling proteins (UCPs) attenuate proton motive force across the inner membrane of mitochondria (IMM) by transporting protons from the intermembrane space into matrix, thus reducing the ATP synthesis rate. UCPs together with adenine nucleotide translocase (ANT) and inorganic phosphate translocase (P_iT) create a subgroup of carriers directly linked to mitochondrial energetics. While its potential role in neuroprotection of neurodegenerative diseases has been speculated, the brain specific neuronal UCP4 is among the least investigated UCPs. This integrative study will focus on the structural features and functional interrelation of UCP4, ANT and P_iT. We designed recombinant versions of these proteins that, using a modified autoinduction method, were overexpressed in the membrane of *Escherichia coli*, extracted with mild detergents, and purified as monomers. Purified proteins, embedded in a shell of bacterial membrane lipids, were then reconstituted in IMM-mimicking phospholipid vesicles for biophysical studies using circular dichroism (CD) and fluorescence spectroscopies. Native-like helical conformations of proteins in the mild detergent octyl glycoside (OG) and phospholipid membranes were confirmed by CD spectroscopy. Interestingly, in agreement with our previous studies, monomeric UCP4 spontaneously self-associated to form tetramers in lipid vesicles. Conversely, ANT and P_iT maintained their molecular forms in both detergent and liposome milieus. Reconstituted proteins in lipid membranes exhibited different levels of proton transport activity in the presence of fatty acids and decreased with the addition of ATP. UCP4 showed significantly higher proton transport rates compared to ANT and P_iT. Research on the functional interrelation between these three carrier proteins, as well as the effect of mitochondrial lipid cardiolipin on their conformational stability and proton transport activity, is in progress in our laboratory.

1944-Pos

Site-specific phosphorylation of VDAC1 decreases its susceptibility to oligomerization

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